Food Analysis
Second Edition

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A Chapman & Hall Food Science Title

Aspen Publishers, Inc., is not affiliated with the American Society of Parenteral and Enteral Nutrition.

Orders: (800) 638-8437
Customer Service: (800) 234-1660

Printed in the United States of America

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Preface and Acknowledgments

The intent of this book is very similar to that described in the Preface to the first edition—a text primarily for undergraduate students majoring in food science, currently studying the analysis of foods. Comments from users of the first edition have convinced me that the book also is a valuable text for persons in the food industry who either do food analysis or interact with analysts. Most of the chapter authors are those from the first edition, but authors have sought to update chapters with new information and techniques, delete less relevant information, and make chapters more useful and readable.

A major change in the second edition is the inclusion of analyses for physical properties. It is recognized that physical properties and chemical composition and characteristics all are important for food quality. Chapters on both chemical and physical properties are not intended as detailed references, but as general introductions to the topics and the techniques. Course instructors may wish to provide more details on a particular topic to students. Chapters focus on principles and applications of techniques. Procedures given are meant to help explain the principles and give some examples, but are not meant to be presented in the detail adequate to actually conduct a specific analysis. As in the first edition, all chapters have summaries and study questions, and key words or phrases are in bold type, to help students focus their studies. Chapters included in the first edition have been updated with new techniques and approaches.

A major effort has been made in revising and adding chapters for the second edition to obtain and utilize input from users of the book. Comments about this second edition from students, instructors, and food industry professionals would be greatly appreciated, so any later edition can better meet their needs.

Of great help to me in editing this edition was an opportunity to work at General Mills, Inc. in Minneapolis, MN on a short sabbatical leave from my teaching and research responsibilities at Purdue University. That experience in the food industry has contributed to making the book more relevant to student training needs. I am indebted to the individuals who made that experience for me possible, and to those who offered suggestions on topics and content to better bring food industry relevance to the book.

I am grateful to new and second-time chapter authors for agreeing to be a part of this project. Many authors have drawn on their experience of teaching students to give chapters the appropriate content, relevance, and ease of use. In addition to those authors, I want to acknowledge the contribution of the previous chapter authors who are now deceased, Drs. Jörg Augustin, Genevieve Christen, Eugenia Davis, and Dick Kleyn. Their contributions to the first edition were of great value to several chapter authors in this second edition. I wish to thank the authors of articles and books, as well as the publishers and industrial companies, for their permission to reproduce materials used here. Special thanks is extended to Melanie King for providing exceptional word processing assistance in the preparation of this book.

S. Suzanne Nielsen

List of Abbreviations

AACC	American Association of Cereal Chemists
AAS	atomic absorption spectroscopy
ABTS	2,2'-azino-d-(3-ethyl-benzthiazoline sulfonate)
AC	alternating current
ADC	analog-to-digital converter
ADP	adenosine-5'-diphosphate
AES	atomic emission spectroscopy
AI	artificial intelligence
AMS	Agricultural Marketing Service
AOAC	Association of Official Analytical Chemists
AOCS	American Oil Chemists' Society
AOM	active oxygen method
APCI	atmospheric pressure chemical ionization
APHA	American Public Health Association
ASCII	American Standard for Information Interchange
ASE	accelerated solvent extraction
ASTM	American Society for Testing Materials
ATTC	American Type Culture Collection
ATP	adenosine-5'-triphosphate
ATR	attenuated total reflectance
ATR–FTIR	attenuated total reflection–Fourier transform infrared
BCA	bicinchoninic acid
BCD	binary coded decimal
BCR	Community Bureau of Reference
Be	Baumé modulus
BGG	bovine gamma globulin
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
BOD	biochemical oxygen demand
BSA	bovine serum albumin
BSDA	*Bacillus sterothermophilis* disk assay
BV	biological value
CAST	calf antibiotic and sulfa test
CDC	Centers for Disease Control
cf	commercial factor
CFR	Code of Federal Regulations
cGMP	Current Good Manufacturing Practices
CI	chemical ionization
CI	confidence interval
CID	charge injection device
CID	Commercial Item Description

CIE	Commission Internationale d'Eclairage
CLND	chemiluminscent nitrogen detector
CMC	critical micelle concentration
CNBr	cyanogen bromide
COD	chemical oxygen demand
C-PER	calculated protein efficiency ratio
CPMG	Carr Purcell Meiboom Gill
CPU	central processing unit
CQC	2,6-dichloroquinonechloroimide
CV	cofficient of variation
CVM	Center for Veterinary Medicine
CW	continuous wave
DAL	defect action level
DC	direct current
DC-PER	discriminant calculated protein efficiency ratio
DE	degree of esterification
DEC	Digital Equipment Corporation
DHHS	Department of Health and Human Services
DMD	D-malate dehydrogenase
DMSO	dimethyl sulfoxide
DMTA	dynamic mechanical thermal analysis
DNFB	1-fluoro-2,4-dinitrobenzene
DNP	dinitrophenyl
DRV	Daily Reference Value
DSC	differential scanning colorimetry
DSHEA	Dietary Supplement Health and Education Act
DTA	differential thermal analysis
DTNB	5,5'-thiobis-2-nitrobenzoic acid
dwb	dry weight basis
ECD	electron capture detector
EDS	energy dispersive spectroscopy
EDTA	ethylenediaminetetraacetic acid
EEC	European Economic Community
EI	electron impact
EIA	enzyme immunoassay
ELCD	electrolytic conductivity detector
ELISA	enzyme linked immunosorbent assay
EMF	electromotive force
EPA	Environmental Protection Agency
EPR	electron paramagnetic resonance
ERH	equilibrium relative humidity
ESA	electrokinetic sonic amplitude
ESI	electrospray interface
ESR	electron spin resonance

ETO	ethylene oxide	HRGC	high resolution gas chromatography
FAME	fatty acid methyl esters	IAs	immunoassays
FAO/WHO	Food and Agricultural Organization/World Health Organization	IC	integrated circuit
		ICP	inductively coupled plasma
		ICP–AES	inductively coupled plasma–atomic emission spectroscopy
FAS	ferrous ammounium sulfate		
FCC	Food Chemicals Codex	ICTA	International Confederation for Thermal Analysis
FDA	Food and Drug Administration		
FD&C	Food, Drug and Cosmetic	IDEA	immobilized digestive enzyme assay
FDNB	1-fluoro-2,4-dinitrobenzene	IFT	Institute of Food Technologists
FFA	free fatty acid	IgG	immunoglobulin G
FIA	fluoroimmunoassay	IMP	integrated pest management
FID	flame ionization detector	IMS	Interstate Milk Shippers
FID	free induction decay	INT	indonitrotetrazolium
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act	IR	infrared
		ISA	ionic strength adjustor
FNB/NAS	Food and Nutrition Board of the National Academy of Sciences	ISE	ion-selective electrode
		ISO	International Organization for Standardization
FPD	flame photometric detector		
FSIS	Food Safety and Inspection Service	IU	International Units
FT	Fourier transform	IUPAC	International Union of Pure and Applied Chemistry
FTC	Federal Trade Commission		
FT–ESR	Fourier transform–electron spin resonance	JECFA	Joint FAO/WHO Expert Committee on Food Additives
FT–ICR	Fourier transform–ion cyclotrons	KFR	Karl Fischer reagent
FTIR	Fourier transform infrared	KFReq	Karl Fischer reagent water equivalence
FT–NMR	Fourier transform–nuclear magnetic resonance	KHP	potassium acid phthalate
		KLH	keyhole limpet hemocyanin
FV	Fruit and Vegetable	LALLS	low-angle laser light scattering
GATT	General Agreement on Tariffs and Trade	LAN	local area network
		LC	liquid chromatography
GC	gas chromatography	LC–MS	liquid chromatography–mass spectroscopy
GC–AED	gas chromatography–atomic emission detector		
		LIMS	laboratory information management system
GC–FTIR	gas chromatography–Fourier transform infrared		
		MAS	magic angle spinning
GC–MS	gas chromatography–mass spectrometry	MCL	maximum contaminant level
		mEq	milliequivalents
GFC	gel-filtration chromatography	MeSCN	methylthiocyanate
GHz	gigahertz	MFL	million fibers per liter
GIPSA	Grain Inspection, Packers and Stockyard Administration	MHz	megahertz
		MPIP	Meat Poultry Inspection Program
GLC	gas–liquid chromatography	MRI	magnetic resonance imaging
GMA	Grocery Manufacturers of America	MRMs	multiresidue methods
GMP	Good Manufacturing Practices	MS	mass spectrometry (or spectrometer)
GOPOD	glucose oxidase/peroxidase	MSCS™	modulated differential scanning calorimeter™
GPC	gel-permeation chromatography		
G6PDH	glucose-6-phosphate dehydrogenase	Ms^n	multiple stages of mass spectrometry
HACCP	Hazard Analysis Critical Control Point	MW	molecular weight
HETP	height equivalent to a theoretical plate	m/z	mass-to-charge ratio
HK	hexokinase	NAD	nicotinamide-adenine dinucleotide
HPLC	high performance liquid chromatography	NADP	nicotinamide-adenine dinucleotide phosphate
HPLC-MS	high performance liquid chromatography–mass spectroscopy	NADPH	reduced NADP
		NCWM	National Conference on Weights and Measures
HPTLC	high performance thin-layer chromatography		
		NIR	near-infrared

NIST	National Institute of Standards and Technology
NLEA	Nutrition Labeling and Education Act
NMFS	National Marine Fisheries Service
NMR	nuclear magnetic resonance
NOAA	National Oceanic and Atmospheric Administration
NPD	nitrogen phosphorus detector or thermionic detector
NPR	net protein ratio
NPU	net protein utilization
NRC	National Research Council
NSSP	National Shellfish Sanitation Program
OCl	organochlorines
ODS	octadecylsilyl
OPA	o-phthalaldehyde
OP	organophosphate
OSI	oil stability index
o/w	oil-in-water
o/w/o	oil-in-water-in-oil
PAGE	polyacrylamide gel electrophoresis
PAM I	Pesticide Analytical Manual, Volume I
PAM II	Pesticide Analytical Manual, Volume II
PCBs	polychlorinated biphenyls
PCR	principal components regression
PDCAAS	protein digestibility—corrected amino acid score
PER	protein efficiency ratio
PFGSE	pulsed field gradient spin echo
pI	isoelectric point
PID	photoionization detector
PLS	partial least squares
PMO	Pasteurized Milk Ordinance
PMT	photomultiplier tube
ppb	parts per billion
PPD	Purchase Product Description
ppm	parts per million
ppt	parts per trillion
PteGln	pteroylglutamate
PUFA	polyunsaturated fatty acids
PVPP	polyvinylpolypyrrolidone
RAC	raw agricultural commodity
RCS	rapid scan correlation
RDI	Reference Daily Intake
RF	radiofrequency
RGB	red green blue
RI	refractive index
RIA	radioimmunoassay
RPAR	Rebuttable Presumption Against Registration
rpm	revolutions per minute
SASO	Saudi Arabian Standards Organization
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SE	standard error of the mean
SEC	size-exclusion chromatography
SEM	scanning electron microscopy
SFC	solid fat content
SFC	supercritical-fluid chromatography
SFE	supercritical fluid extraction
SFE–GC	supercritical fluid extraction–gas chromatography
SFI	solid fat index
SIM	selected ion monitoring
S/L	solid/liquid
SNF	solids-not-fat
SNIF-NMR	site-specific natural isotope fractionation - NMR
SPE	solid-phase extraction
SPME	solid-phase microextraction
SO	sulfite oxidase
SQC	statistical quality control
SRMs	single residue methods
STOP	swab test on premises
TBA	thiobarbituric acid
TBARS	TBA reactive substances
TCD	thermal conductivity detector
TE	tocopherol equivalents
TEMED	tetramethylethylenediamine
TGA	thermogravimetric analysis
TIC	total ion current
TLC	thin-layer chromatography
TMA	thermomechanical analysis
TMCS	trimethylchlorosilane
TMS	trimethylsilyl
TNBS	trinitrobenzenesulphonic acid
TOC	total organic carbon
TOF	time-of-flight
TPA	texture profile analysis
TS	total solids
TS–MS	thermospray–mass spectrometry
TSP	thermospray
TSS	total soluble solids
TSUSA	Tariff Schedules of the United States of America
USCS	United States Customs Service
USDA	United States Department of Agriculture
US RDA	United States Recommended Dietary Allowance
UV	ultraviolet
UV-Vis	ultraviolet-visible
Vis	visible
VPP	vegetable protein product
w/o/w	water-in-oil-in-water
wwb	wet weight basis

part

1

General Information

1
chapter

Introduction to Food Analysis

S. Suzanne Nielsen

1.1 INTRODUCTION

Investigations in food science and technology, whether by the food industry, governmental agencies, or universities, often require determination of food composition and characteristics. Trends and demands of consumers, the food industry, and national and international regulations challenge food scientists as they work to monitor food composition and to ensure the quality and safety of the food supply. All food products require analysis as part of a quality management program throughout the development process, through production, and after a product is in the market. The chemical composition and physical properties of foods are used to determine the nutritive value, functional characteristics, and acceptability of the food product. The nature of the sample and the specific reason for the analysis commonly dictate the choice of analytical methods. Speed, precision, accuracy, and durability often are key factors in this choice. Validation of the method for the specific food matrix being analyzed is necessary to ensure usefulness of the method. The success of any analytical method relies on the proper selection and preparation of the food sample, carefully performing the analysis, and doing the appropriate calculations and interpretation of the data. Methods of analysis developed and endorsed by several nonprofit scientific organizations allow for standardized comparisons of results between different laboratories, and for evaluation of less standard procedures. Such official methods are critical in the analysis of foods, to ensure that they meet the legal requirements established by governmental agencies. Government regulations and international standards most relevant to the analysis of foods are mentioned here but covered in more detail in Chapter 2, and nutrition labeling regulations in the United States are covered in Chapter 3. Internet addresses for many of the organizations and government agencies discussed are given at the end of this chapter.

1.2 TRENDS AND DEMANDS

1.2.1 Consumers

Consumers have many choices regarding their food supply, so they can be very selective about the products they purchase. They demand a wide variety of products that are of high quality, safe, nutritious, and offer a good value. Many consumers are interested in the relationship between diet and health, so they use nutrient content and health claim information from food labels to make purchase choices. These factors create a challenge for the food industry and for its employees. For example, the demand for foods with lower fat content challenges food scientists to develop food products that contain fat content claims (e.g., free, low,

reduced) and certain health claims (e.g., the link between dietary fat and cancer; dietary saturated fat and cholesterol and risk of coronary heart disease). Analytical methods to determine and characterize fat content provide the data necessary to justify these statements and claims. Use of fat substitutes in product formulations makes possible many of the lower fat foods, but these fat substitutes can create challenges in the accurate measurement of fat content (1, 2).

1.2.2 Food Industry

To compete in the marketplace, food companies must produce foods that meet the demands of consumers as described previously. Management of product quality by the food industry is of increasing importance, beginning with the raw ingredients and extending to the final product eaten by the consumer. Analytical methods must be applied across the entire food supply chain to achieve the desired final product quality. Downsizing in response to increasing competition in the food industry often has pushed the responsibility for ingredient quality to the suppliers. Many companies have select suppliers, on whom they rely to perform the analytical tests to ensure compliance with detailed ingredient specifications.

Traditional quality control and quality assurance concepts are only a portion of a comprehensive quality management system. Food industry employees responsible for quality management work together in teams with other individuals in the company responsible for product development, production, marketing, and regulatory and consumer affairs.

Analytical information must be obtained, assessed, and integrated with other relevant information about the food system to address quality-related problems. Making appropriate decisions depends on having a knowledge of the analytical methods and equipment utilized to obtain the data on quality characteristics. To design experiments in product and process development, one must know the operating principles and capabilities of the analytical methods used to assess results of the experiments to be conducted. Upon completion of these experiments, one must critically evaluate the analytical data collected to determine whether product reformulation is needed or what parts of the process need to be modified for future tests. The situation is similar in the research laboratory, where knowledge of analytical techniques is necessary to design experiments, and the evaluation of data obtained determines the next experiments to be conducted.

1.2.3 Government Regulations and International Standards and Policies

To market safe, high quality foods effectively in a national and global marketplace, food companies must

pay increasing attention to government regulations and guidelines, and to the policies and standards of international organizations. Food scientists must be aware of these regulations, guidelines, and policies related to food safety and quality, and know the implications for food analysis. Government regulations and guidelines in the United States relevant to food analysis, include nutrition labeling regulations (Chapter 3), Good Manufacturing Practice (GMP) regulations (Chapter 2), and Hazard Analysis Critical Control Point (HACCP) systems (Chapter 2). The HACCP concept has been adopted not only by the United States Food and Drug Administration (FDA) and other federal agencies in the United States but also by the Codex Alimentarius Commission, an international organization that has become a major force in world food trade. Codex is described in Chapter 2, along with other organizations active in developing international standards and safety practices relevant to food analysis that affect the import and export of raw agricultural commodities and processed food products.

1.3 TYPES OF SAMPLES ANALYZED

Chemical analysis of foods is an important part of a quality assurance program in food processing, from ingredients and raw materials, through processing, to the finished products (3–7). Chemical analysis also is important in formulating and developing new products, evaluating new processes for making food products, and in identifying the source of problems with unacceptable products (Table 1-1). For each type of product to be analyzed, it may be necessary to determine either just one or many components. The nature of the sample and the way in which the information obtained will be used may dictate the specific method of analysis. For example, process control samples are usually analyzed by rapid methods, whereas nutritive value information for nutrition labeling generally requires the use of more time consuming methods of analysis endorsed by scientific organizations. Critical questions, including those listed in Table 1-1, can be answered by analyzing various types of samples in a food processing system.

1.4 STEPS IN ANALYSIS

1.4.1 Select and Prepare Sample

In analyzing food samples of the types described previously, all results depend on obtaining a representative sample and converting the sample to a form that can be analyzed. Neither of these is as easy as it sounds! Sampling and sample preparation are covered in detail in Chapter 5.

| 1-1 table | Types of Samples Analyzed in a Quality Assurance Program for Food Products |

Sample Type	Critical Questions
Raw materials	Do they meet your specifications? Do they meet required legal specifications? Will a processing parameter have to be modified because of any change in the composition of raw materials? Are the quality and composition the same as for previous deliveries? How does the material from a potential new supplier compare to that from the current supplier?
Process control samples	Did a specific processing step result in a product of acceptable composition or characteristics? Does a further processing step need to be modified to obtain a final product of acceptable quality?
Finished product	Does it meet the legal requirements? What is the nutritive value, so that label information can be developed? Or is the nutritive value as specified on an existing label? Does it meet product claim requirements (e.g., "low fat")? Will it be acceptable to the consumer? Will it have the appropriate shelf life?
Competitor's sample	What are its composition and characteristics? How can we use this information to develop new products?
Complaint sample	How do the composition and characteristics of a complaint sample submitted by a customer differ from a sample with no problems?

Adapted and updated from (8, 9).

1.4.2 Perform the Assay

Performing the assay is unique for each component or characteristic to be analyzed and may be unique to a specific type of food product. Single chapters in this book address sampling and sample preparation (Chapter 5) and data handling (Chapter 4), while the remainder of the book addresses the step of actually performing the assay. The descriptions of the various specific procedures are meant to be overviews of the methods. For guidance in actually performing the assays, details regarding chemicals, reagents, apparatus, and step-by-step instructions are found in the referenced books and articles.

1.4.3 Calculate and Interpret the Results

To make decisions and take action based on the results obtained from performing the assay that determined

the composition or characteristics of a food product, one must make the appropriate calculations to interpret the data correctly. Data handling is covered in Chapter 4.

1.5 CHOICE AND VALIDITY OF A METHOD

1.5.1 Characteristics of the Method

Numerous methods often are available to assay food samples for a specific characteristic or component. To select or modify methods used to determine the chemical composition and characteristics of foods, one must be familiar with the principles underlying the procedures and the critical steps. Certain properties of methods and criteria described in Table 1-2 are useful to evaluate the appropriateness of a method in current use or a new method being considered.

1.5.2 Objective of the Method

Selection of a method depends largely on the objective of the measurement. For example, methods used for rapid on-line processing measurements may be less accurate than official methods (see section 1.6) used for nutritional labeling purposes. Methods referred to as reference, definitive, official, or primary are most applicable in a well equipped and staffed analytical lab. The more rapid secondary or field methods may be more applicable on the manufacturing floor in a food processing facility. For example, refractive index may be used as a rapid, secondary method for sugar analysis (see Chapters 8 and 11), with results correlated to those of the primary method, high performance liquid chromatography (HPLC) (see Chapters 11 and 32). Moisture content data for a product being developed in the pilot plant may be obtained quickly with a moisture balance unit that has been calibrated using a more time-consuming hot air oven method (see Chapter 8).

1.5.3 Consideration of Food Composition and Characteristics

The performance of many analytical methods is affected by the food matrix, i.e., its chemical composition. For example, high fat or high sugar foods can cause different types of interferences than low fat or low sugar foods. Digestion procedures and extraction steps necessary for accurate analytical results can be very dependent on the food matrix. The complexity of various food systems often requires having not just one technique available for a specific food component, but multiple techniques and procedures, as well as the knowledge about which to apply to a specific food matrix.

A task force of AOAC International, formerly known as the Association of Official Analytical Chemists (AOAC), suggested a "triangle scheme" for dividing foods into matrix categories (10–12) (Fig. 1-1). The apexes of the triangle contain food groups that were either 100% fat, 100% protein, or 100% carbohydrate. Foods were rated as "high," "low," or "medium" based on levels of fat, carbohydrate, and proteins, which are the three nutrients expected to have the strongest effect on analytical method performance. This created nine possible combinations of high, medium, and low levels of fat, carbohydrate, and protein. Complex foods were positioned spatially in the triangle according to their content of fat, carbohydrate, and protein, on a normalized basis (i.e., fat, carbohydrate, and protein normalized to total 100%). General analytical methods ideally would be geared to handle each of the nine combinations, replacing more numerous matrix-dependent methods developed for specific foods. For example, using matrix-dependent methods, one method might be applied to potato chips and chocolates, which are both low protein, medium fat, medium carbohydrate foods, but another might be required for a high protein, low fat, high carbohydrate food such as nonfat dry milk (11).

1.5.4 Validity of the Method

Numerous factors affect the usefulness and validity of the data obtained using a specific analytical method. One must consider certain characteristics of any method, such as specificity, precision, accuracy, and sensitivity (see Table 1-2 and Chapter 4). However, one also must consider how the variability of data from the method for a specific characteristic compares to differences detectable and acceptable to a consumer, and the variability of the specific characteristic inherent in processing of the food. One must consider the nature of the samples collected for the analysis, how representative the samples were of the whole, and the number of samples analyzed (Chapter 5). One must ask whether details of the analytical procedure were followed adequately, such that the results are accurate, repeatable, and comparable to data collected previously. For data to be valid, equipment to conduct the analysis must be standardized and appropriately used, and the performance limitations of the equipment recognized.

A major consideration for determining method validity is the analysis of materials used as controls, often referred to as standard reference materials or check samples. Standard reference materials can be obtained in the United States from the National Institute of Standards and Technology (NIST), in Canada from the Center for Land and Biological Resource Research, and in Belgium from the Community Bureau of Reference (BCR). Numerous organizations offer

1-2 table   Criteria for Choice Food Analysis Methods

Characteristic	Critical Questions	Characteristic	Critical Questions
Inherent properties			Is the method very sensitive to slight or moderate changes in the reagents?
• Specificity	Is the property being measured the same as that claimed to be measured? What steps are being taken to ensure a high degree of specificity?	• Equipment	Do you have the appropriate equipment? Are personnel competent to operate equipment?
• Precision	What is the precision of the method? Is there within-batch, batch-to-batch, or day-to-day variation? What step in the procedure contributes the greatest variability?	• Cost	What is the cost in terms of equipment, reagents, and personnel?
• Accuracy	How does the new method compare in accuracy to the old or a standard method? What is the percent recovery?	*Usefulness*	
		• Time required	How fast is it? How fast does it need to be?
		• Reliability	How reliable is it from the standpoints of precision and stability?
Applicability of method to laboratory		• Need	Does it meet a need or better meet a need? Is any change in method worth the trouble of the change?
• Sample size	How much sample is needed? Is it too large or too small to fit your needs? Does it fit your equipment and/or glassware?	*Personnel*	
		• Safety	Are special precautions necessary?
• Reagents	Can you properly prepare them? What equipment is needed? Are they stable? For how long and under what conditions?	• Procedures	Who will prepare the written description of the procedures and reagents? Who will do any required calculations?
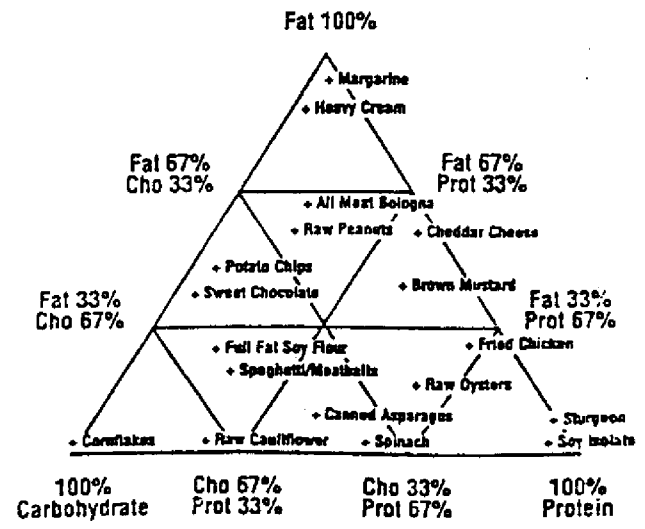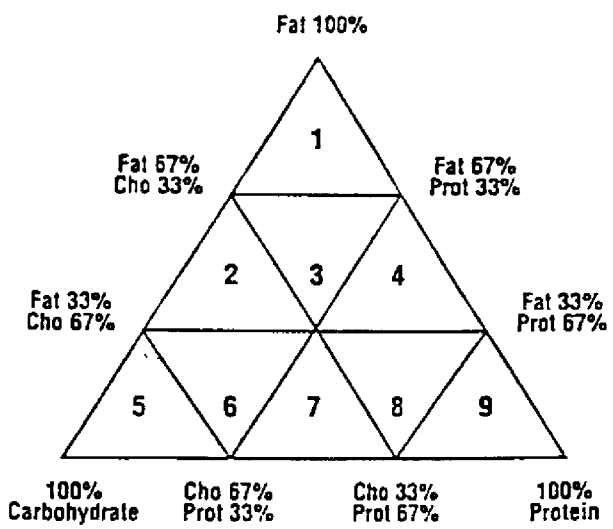
1-1 figure Schematic layout of food matrixes based on protein, fat, and carbohydrate content, excluding moisture and ash. Reprinted with permission from (12), *Inside Laboratory Management*, September 1997, p. 33. Copyright 1997, by AOAC International.

check sample services that provide test samples to evaluate the reliability of a method (13). For example, the American Association of Cereal Chemists (AACC) has a check sample service in which a subscribing laboratory receives specifically prepared test samples from AACC. The subscribing laboratory performs the specified analyses on the samples and returns the results to AACC. The AACC then provides a statistical evaluation of the analytical results and compares the subscribing laboratory's data with those of other laboratories to inform the subscribing laboratory of its degree of accuracy. The AACC offers check samples such as flours and semolina for the analysis of vitamins and minerals, sugars, sodium, total dietary fiber, soluble and insoluble dietary fiber, β-glucan, near infrared analyses, sanitation, and microbiology.

The American Oil Chemists' Society (AOCS) has a check sample program for oilseeds, oilseed meals, marine oils, aflatoxin, trans fatty acids, oils/fats, and various oil and fat constituents. Laboratories from many countries participate in the program to check the accuracy of their work, their reagents, and their laboratory apparatus against the statistical norm derived from the group data.

Standard reference materials are important tools to ensure reliable data. However, such materials need not necessarily be obtained from outside organizations. Control samples internal to the laboratory can be prepared by carefully selecting an appropriate type of sample, gathering a large quantity of the material, mixing and preparing to ensure homogeneity, packaging the sample in small quantities, storing the samples appropriately, and routinely analyzing the control sample when test samples are analyzed. Whatever the standard reference materials used, these should match closely the matrix of the samples to be analyzed by a specific method.

1.6 OFFICIAL METHODS

The choice of method for a specific characteristic or component of a food sample is often made easier by the availability of official methods. Several nonprofit scientific organizations have compiled and published these methods of analysis for food products, which have been carefully developed and standardized. They allow for comparability of results between different laboratories that follow the same procedure, and for evaluating results obtained using new or more rapid procedures.

1.6.1 AOAC International

AOAC International (formerly the Association of Official Analytical Chemists) is an organization begun

in 1884 to serve the analytical methods needs of government regulatory and research agencies. The goal of AOAC International is to provide methods that will be fit for their intended purpose, i.e., will perform with the necessary accuracy and precision under usual laboratory conditions.

This volunteer organization functions as follows:

1. Methods of analysis from published literature are selected or new methods are developed by AOAC International volunteers.
2. Methods are collaboratively tested using multilaboratory studies in volunteers' laboratories.
3. Methods are given a multilevel peer review by expert scientists, and if found acceptable, are adopted as official methods of analysis.
4. Adopted methods are published in the *Official Methods of Analysis*, which covers a wide variety of assays related to foods, drugs, cosmetics, agriculture, forensic science, and products affecting public health and welfare.
5. AOAC International publishes manuals, methods compilations in specific areas of analysis, monographs, and the monthly magazine, *Inside Laboratory Management*.
6. AOAC International conducts training courses of interest to analytical scientists and other laboratory personnel.

Methods validated and adopted by AOAC International and the data supporting the method validation are published in the *Journal of AOAC International*. Such methods must be successfully validated in a formal interlaboratory collaborative study before being accepted as an official first action method by AOAC International. Details of the validation program (e.g., number of laboratories involved, samples per level of analyte, controls, control samples, and the review process) are given in the front matter of the AOAC International's *Official Methods of Analysis*. First action methods are subject to scrutiny and general testing by other scientists and analysts for at least two years before final action adoption. Adopted first action and final action methods are compiled in books published and updated every four to five years as the *Official Methods of Analysis* (14) of AOAC International. Supplements to the book are published yearly and contain new methods and revisions to current methods. The *Official Methods of Analysis* of AOAC International includes methods appropriate for a wide variety of products and other materials (Table 1-3). These methods often are specified by the FDA with regard to legal requirements for food products. They are generally the methods followed by the FDA and the Food Safety and Inspection Service (FSIS) of the USDA (United States

1-3
table   Table of Contents of 1995 Official Methods of Analysis of AOAC International (14)

Chapter	Title	Chapter	Title
1	Agriculture Liming Materials	26	Distilled Liquors
2	Fertilizers	27	Malt Beverages and Brewing Materials
3	Plants	28	Wines
4	Animal Feed	29	Nonalcoholic Beverages and Concentrates
5	Drugs in Feeds	30	Coffee and Tea
6	Disinfectants	31	Cacao Bean and Its Products
7	Pesticide Formulations	32	Cereal Foods
8	Hazardous Substances	33	Dairy Products
9	Metals and Other Elements at Trace Levels in Foods	34	Eggs and Egg Products
10	Pesticide and Industrial Chemical Residues	35	Fish and Other Marine Products
12	Microchemical Methods	37	Fruits and Fruit Products
13	Radioactivity	38	Gelatin, Dessert Preparations, and Mixes
14	Veterinary Analytical Toxicology	39	Meat and Meat Products
15	Cosmetics	40	Nuts and Nut Products
16	Extraneous Materials: Isolation	41	Oils and Fats
17	Microbiological Methods	42	Vegetable Products, Processed
18	Drugs: Part I	43	Spices and Other Condiments
19	Drugs: Part II	44	Sugars and Sugar Products
20	Drugs: Part III	45	Vitamins and Other Nutrients
21	Drugs: Part IV	46	Color Additives
22	Drugs: Part V	47	Food Additives: Direct
23	Drugs and Feed Additives in Animal Tissues	48	Food Additives: Indirect
24	Forensic Sciences	49	Natural Toxins
25	Baking Powders and Baking Chemicals	50	Infant Formulas and Medical Diets

Department of Agriculture) to check the nutritional labeling information on foods and to check foods for the presence or absence of undesirable residues or residue levels.

1.6.2 American Association of Cereal Chemists

The American Association of Cereal Chemists (AACC) publishes a set of approved laboratory methods, applicable mostly to cereal products (Table 1-4). The AACC process of adopting the *Approved Methods of Analysis* (15) is consistent with the process used by the AOAC International and American Oil Chemists' Society (AOCS). Approved methods of the AACC are continuously reviewed, critiqued, and updated. They are printed in a looseleaf format, contained in ring binders. Supplements containing new and revised procedures are provided annually.

1.6.3 American Oil Chemists' Society

The American Oil Chemists' Society (AOCS) publishes a set of official methods and recommended practices, applicable mostly to fat and oil analysis (Table 1-5) (16). AOCS is a widely used methodology source on the subjects of edible fats and oils, oilseeds and oilseed proteins, soaps and synthetic detergents,

industrial fats and oils, fatty acids, oleochemicals, glycerin, and lecithin.

1.6.4 Other Endorsed Methods

Standard Methods for the Examination of Dairy Products (17), published by the American Public Health Association, includes methods for the chemical analysis of products (Table 1-6). *Standard Methods for the Examination of Water and Wastewater* (18) is published jointly by the American Public Health Association, American Water Works Association, and the Water Environment Federation. *Food Chemicals Codex* (19), published by the Food and Nutrition Board of the National Research Council/National Academy of Science, contains methods for the analysis of certain food additives. The American Spice Trade Association (20), the Infant Formula Council (21), and the Corn Refiners Association (22) are among the organizations that publish standard methods for the analysis of their respective products.

1.7 SUMMARY

Food scientists and technologists determine the chemical composition and physical characteristics of foods routinely as part of their quality management, product

1-4 table	Table of Contents of 1995 Approved Methods of the American Association of Cereal Chemists (15)

1-5 table	Table of Contents of 1996 Official Methods and Recommended Practices of the American Oil Chemists' Society (16)

als to ensure quality results. Rapid methods used for quality assessment in a production facility may be less accurate but much faster than official methods used for nutritional labeling. Endorsed methods for the chemical analyses of foods have been compiled and published by AOAC International, American Association of Cereal Chemists, American Oil Chemists' Society, and certain other nonprofit scientific organizations. These methods allow for comparison of results between different laboratories and for evaluation of new or more rapid procedures.

1.8 STUDY QUESTIONS

1. Identify six reasons you might need to determine certain chemical characteristics of a food product as part of a quality management program.
2. You are considering the use of a new method to measure Compound X in your food product. List six factors you will consider before adopting this new method in your quality assurance laboratory.
3. In your work at a food company, you mentioned to a coworker something about the *Official Methods of Analysis* published by AOAC International. The coworker asks you what the term "AOAC" refers to, what AOAC International does, and what the *Official Methods of Analysis* is. Answer your coworker's questions.
4. For each type of product listed below, identify a publication in which you can find standard methods of analysis appropriate for the product:
   a. ice cream
   b. enriched flour
   c. wastewater (from food processing plant)
   d. margarine
   e. ground cinnamon

development, or research activities. For example, the types of samples analyzed in a quality management program of a food company can include raw materials, process control samples, finished products, competitors' samples, and consumer complaint samples. Consumer, food industry, and government concern for food quality and safety has increased the importance of analyses that determine composition and critical product characteristics.

To successfully base decisions on results of any analysis, one must correctly conduct all three major steps in the analysis: (1) select and prepare samples, (2) perform the assay, and (3) calculate and interpret the results. The choice of analysis method is usually based on the objective of the analysis, characteristics of the method itself (e.g., specificity, accuracy, precision, speed, cost of equipment, and training of personnel), and the food matrix involved. Validation of the method is important, as is the use of standard reference materi-

1-6 table — Contents of Chapter 15 on Chemical and Physical Methods in Standard Methods for the Examination of Dairy Products (17)

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21. Infant Formula Council. 1995. *Methods of Analysis Manual for Infant Formulas.* (updated periodically). Infant Formula Council, Atlanta, GA.

22. Corn Refiners Association. 1997. *Standard Analytical Methods of the Member Companies of Corn Industries Research Foundation.* Corn Refiners Association, Inc., Washington, DC.

1.10 RELEVANT INTERNET ADDRESSES

American Association of Cereal Chemists
    http://www.scisoc.org:80/aacc/
American Oil Chemists' Society
    http://www.aocs.org/
American Public Health Association
    http://www.apha.org/
AOAC International
    http://www.aoac.org

Code of Federal Regulations
    http://www.access.gpo.gov/nara/cfr/cfr-table-search.html
Codex Alimentarius Commission
    http://www.fao.org/waicent/faoinfo/economic/esn/codex/codex.htm
Food Chemicals Codex
    http://wvw2.nas.edu/codex
Food and Drug Administration
    http://www.fda.gov
    Center for Food Safety & Applied Nutrition
        http://vm.cfsan.fda.gov/list.html
        Current Good Manufacturing Practices
            http://vm.cfsan.fda.gov/~lrd/part110t.txt
        Food Labeling and Nutrition
            http://vm.cfsan.fda.gov/label.html
        Hazard Analysis Critical Control Point
            http://vm.cfsan.fda.gov/~lrd/haccpsub.html
National Institute of Standards and Technology
    http://www.nist.gov
U.S. Department of Agriculture
    http://www.usda.gov
    Food Safety and Inspection Service
        http://www.usda.gov/fsis
        HACCP/Pathogen Reduction
            http://www.usda.gov/agency/fsis/imphaccp.htm

2
chapter

United States Government Regulations and International Standards Related to Food Analysis

S. Suzanne Nielsen

2.1 INTRODUCTION

Knowledge of government regulations relevant to the chemical analysis of foods is extremely important to persons working in the food industry. Federal laws and regulations reinforce the efforts of the food industry to provide wholesome foods, to inform consumers about the nutritional composition of foods, and to eliminate economic frauds. In some cases, they dictate what ingredients a food must contain, what must be tested, and the procedures used to analyze foods for safety factors and quality attributes. This chapter describes the United States federal regulations related to the composition of foods. The reader is referred to references 1–6 for comprehensive coverage of United States food laws and regulations. Many of the regulations referred to in this chapter are published in the various Titles of the Code of Federal Regulations (CFR) (7). This chapter also includes information about food standards and safety practices established by international organizations. Internet addresses are given at the end of this chapter for many of the government agencies, organizations, and documents discussed.

2.2 UNITED STATES FEDERAL REGULATIONS AFFECTING FOOD COMPOSITION

2.2.1 United States Food and Drug Administration

The Food and Drug Administration (FDA) is a United States government agency within the Department of Health and Human Services (DHHS). The FDA is responsible for regulating, among other things, the safety of foods, cosmetics, drugs, medical devices, biologicals, and radiological products. It acts under laws passed by the United States Congress to monitor the affected industries and ensure the consumer of the safety of such products.

2.2.1.1 Legislative History

2.2.1.1.1 Food and Drug Act of 1906 The Food and Drug Act of 1906, reenacted in 1907 to extend the provisions for an indefinite period, was the first federal law governing the food supply in the United States. It made illegal the interstate commerce of misbranded or adulterated manufactured or natural foods, beverages, drugs, medicines, or stock feeds. It stated that only substances not likely to render a food injurious to health could be added to foods.

The United States Department of Agriculture (USDA) was responsible for administering the 1906 Act until 1931, when the FDA was created to administer it. Although the FDA was originally a part of the

USDA, it now operates within the DHHS. The USDA and then the FDA were both ineffective in enforcing the 1906 Act because the Act lacked fines or other penalties for violators. This led to eventual passage of the Federal Food, Drug, and Cosmetic (FD&C) Act of 1938.

2.2.1.1.2 Federal Food, Drug, and Cosmetic Act of 1938 The FD&C Act of 1938 broadened the scope of the 1906 Act, intending to assure consumers that foods are safe and wholesome, produced under sanitary conditions, and packaged and labeled truthfully. The law further defined and set regulations on adulterated and misbranded foods. The FDA was given power to seize illegal products and to imprison and fine violators. An important part of the 1938 Act relevant to food analysis is the section that authorizes food definitions and standards of identity, as further described below.

2.2.1.1.3 Amendments and Additions to the 1938 FD&C Act The 1938 FD&C Act has been amended several times to increase its power. The Miller Pesticide Amendment was added in 1954 to specify the acceptable amount of pesticide residues an fresh fruits, vegetables, and other raw agricultural products when they enter the marketplace. This Amendment, then under the authority of the FDA, is now administered by the Environmental Protection Agency (EPA).

The Food Additives Amendment to the 1938 Act was enacted in 1958. It was designed to protect the health of consumers by requiring a food additive to be proven safe before addition to a food and to permit the food industry to use food additives that are safe at the intended level of use. The highly controversial Delaney Clause, attached as a rider to this amendment, prohibits the FDA from setting any tolerance level as a food additive for substances known to be carcinogenic.

The Color Additives Amendment, passed in 1960, defines color additives, sets rules for both certified and uncertified colors, provides for the approval of color additives that must be certified or are exempt from certification, and empowers the FDA to list color additives for specific uses and set quantity limitations. Similar to the Food Additives Amendment, the Color Additives Amendment contains a Delanev Clause.

The Nutrition Labeling and Education Act of 1990 (NLEA), described further in Chapter 3, made nutrition labeling mandatory on most food products under FDA jurisdiction, and established definitions for health and nutrient claims. The NLEA emphasized the relationship between diet and health, and provided consumers a means to choose foods based on complete and truthful label information.

The Dietary Supplement Health and Education Act (1994) (DSHEA) changed the definition and regulations for dietary supplements from those in the FD&C Act and in acts relevant to dietary supplements

passed prior to 1994. The DSHEA defined supplements as "dietary ingredients" (defined in specific but broad terms), set criteria to regulate claims and labeling, and established government agencies to handle regulation. Classified now as "dietary ingredients" rather than by the previously used term "food additives," dietary supplements are not subject to the Delaney Clause of the FD&C Act. Regulations for dietary supplements permit claims not allowed for traditional foods. Control and regulation of dietary supplements have been separated from those for traditional foods.

The Food Quality Protection Act (1996) amended both the FD&C Act and the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), as further described in section 2.2.51.

2.2.1.1.4 Other FDA Regulations

The FDA has developed many administrative rules, guidelines, and action levels, in addition to the regulations described above, to implement the FD&C Act of 1938. Most of them are published in Title 21 of the CFR. They include the Good Manufacturing Practice (GMP) regulations (21 CFR 110), regulations regarding food labeling (21 CFR 101), recall guidelines (21 CFR 7.40), and nutritional quality guidelines (21 CFR 104). The food labeling regulations include nutritional labeling requirements and guidelines, and specific requirements for nutrient content, health claims, and descriptive claims (discussed in Chapter 3).

The FDA administers several other federal statutes related to foods. The Fair Packaging and Labeling Act of 1966 requires that the net weight of a food product, among other information, be accurately stated on the label in a specific manner. Among the provisions enforced by the FDA in the Public Health Service Act of 1944 are the safety of pasteurized milk and shellfish, as discussed in sections 2.3 and 2.4, respectively. The importation of milk and cream into the United States is regulated by the Import Milk Act of 1927 for economic and public health considerations. Certain aspects of the amended Federal Meat Inspection Act of 1967 and the amended Poultry Products Inspection Act of 1957 (discussed in sections 2.2.2.1.1 and 2.2.2.2.3) are administered by the FDA.

A comprehensive collection of federal laws, guidelines, and regulations relevant to foods and drugs has been published by the Food and Drug Law Institute (2). The staff of *Food Chemical News* has prepared the "FDA Food Enforcement Handbook," a compilation of FDA enforcement guides relevant to food processors (3). With increasing responsibility being placed on food processors and regulatory agencies to ensure the safety of foods eaten by consumers, FDA has placed considerable emphasis on Good Manufacturing Practice regulations and on Hazard Analysis Critical Control Point (HACCP) systems. HACCP is an important com-

ponent of an interagency initiative to reduce the incidence of foodborne illness, and includes the FDA, USDA, Environmental Protection Agency (EPA), and the Centers for Disease Control (CDC) (8). Both GMP and HAACP systems emphasize the importance of preventing hazards, to avoid problems associated with detecting hazards in foods.

The GMP regulations, legally based on the FD&C Act, but not established as a proposed rule until 1969, are designed to prevent adulterated food in the marketplace (9). The GMP regulations define requirements for acceptable sanitary operation in food plants and include the following relevant to food processing:

1. General provisions that define and interpret the detailed regulations;
2. Requirements and expectations for maintaining grounds, building, and facilities;
3. Requirements and expectations for design, construction, and maintenance of equipment;
4. Requirements for production and process controls; and
5. Defect action levels (DALs) for natural and unavoidable defects.

In addition to general GMPs (21 CFR 110), specific GMPs exist for thermally processed low-acid canned foods (21 CFR 113), acidified foods (21 CFR 114), and bottled drinking water (21 CFR 129).

Unlike GMP regulations, HACCP is a system developed and implemented by a food processor, originally designed to produce zero defect (no hazard) food for astronauts to consume on space flights (10). The FDA and USDA have adopted the HACCP concept in certain of their inspection programs. An effective HACCP program has the following components:

1. Determine potential hazards in each process.
2. Identify critical control points.
3. Establish control limits for each critical control point.
4. Establish procedures to monitor control points.
5. Establish corrective actions when limits of control point are exceeded.
6. Establish appropriate system of record keeping.
7. Establish program to verify efficacy of program.

While GMPs and HACCP programs are based largely on microbiological concerns, certain chemical and physical tests (e.g., inactivation of toxic constituents, presence of extraneous matter, metal detection) are often necessary to ensure the safety of foods.

2.2.1.2 Food Definitions and Standards

The food definitions and standards established by the FDA are published in 21 CFR 100–169 and include stan-

dards of identity, quality, and fill. The standards of identity, which have been set for a wide variety of food products, are most relevant to the chemical analysis of foods because they specifically establish which ingredients a food must contain. They limit the amount of water permitted in certain products. The minimum levels for expensive ingredients are often set, and maximum levels for inexpensive ingredients are sometimes set. The kind and amount of certain vitamins and minerals that must be present in foods labeled "enriched" are specified. The standards of identity for some foods include a list of optional ingredients. The standard of identity for sour cream (21 CFR 131.160) is given in Fig. 2-1. Table 2-1 summarizes the standards of identity relevant to food analysis for a number of other foods. Note that the standard of identity often includes the recommended analytical method for determining chemical composition.

Although standards of quality and fill are less related to the chemical analysis of foods than are standards of identity, they are important for economic and quality control considerations. Standards of quality, established by the FDA for some canned fruits and vegetables, set minimum standards and specifications for factors such as color, tenderness, weight of units in the container, and freedom from defects. The standards of fill established for some canned fruits and vegetables, tomato products, and seafood state how full a container must be to avoid consumer deception.

2.2.1.3 Inspection and Enforcement

The FDA has broadest regulatory authority over most foods (generally, all foods other than meat, poultry, eggs; water supplies; imported foods). However, the FDA shares responsibilities with other regulatory agencies for certain foods, as described in later sections of this chapter. The FDA has responsibility for enforc-

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§ 131.160 Sour cream.

(a) *Description.* Sour cream results from the souring, by lactic acid producing bacteria, of pasteurized cream. Sour cream contains not less than 18 percent milkfat; except that when the food is characterized by the addition of nutritive sweeteners or bulky flavoring ingredients, the weight of the milkfat is not less than 18 percent of the remainder obtained by subtracting the weight of such optional ingredients from the weight of the food; but in no case does the food contain less than 14.4 percent milkfat. Sour cream has a titratable acidity of not less than 0.5 percent, calculated as lactic acid.

(b) *Optional ingredients.* (1) Safe and suitable ingredients that improve texture, prevent syneresis, or extend the shelf life of the product.

(2) Sodium citrate in an amount not more than 0.1 percent may be added prior to culturing as a flavor precursor.

(3) Rennet.

(4) Safe and suitable nutritive sweeteners.

(5) Salt.

(6) Flavoring ingredients, with or without safe and suitable coloring, as follows:

(i) Fruit and fruit juice (including concentrated fruit and fruit juice).

(ii) Safe and suitable natural and artificial food flavoring.

(c) *Methods of analysis.* Referenced methods in paragraph (c) (1) and (2) of this section are from "Official Methods of Analysis of the Association of Official Analytical Chemists," 13th Ed. (1980), which is incorporated by reference. Copies may be obtained from the Association of Official Analytical Chemists, 2200 Wilson Blvd., Suite 400, Arlington, VA 22201-3301, or may be examined at the Office of the Federal Register, 800 North Capitol Street, NW., suite 700, Washington, DC.

(1) Milkfat content—"Fat—Official Final Action," section 16.172.

(2) Titratable acidity—"Acidity—Official Final Action," section 16.023.

(d) *Nomenclature.* The name of the food is "Sour cream" or alternatively "Cultured sour cream". The full name of the food shall appear on the principal display panel of the label in type of uniform size, style, and color. The name of the food shall be accompanied by a declaration indicating the presence of any flavoring that characterizes the product, as specified in § 101.22 of this chapter. If nutritive sweetener in an amount sufficient to characterize the food is added without addition of characterizing flavoring, the name of the food shall be preceded by the word "sweetened".

(e) *Label declaration.* Each of the ingredients used in the food shall be declared on the label as required by the applicable sections of parts 101 and 130 of this chapter.

[42 FR 14360, Mar. 15, 1977, as amended at 47 FR 11824, Mar. 19, 1982; 49 FR 10092, Mar. 19, 1984; 54 FR 24893, June 12, 1989; 58 FR 2891, Jan. 6, 1993]

2-1 figure Standard of identity for sour cream. [From 21 CFR 131.160 (1997).]

2-1 table Selected Chemical Composition Requirements of Some Foods with Standards of Identity

Section in 21 CFR[1]	Food Product	Requirement	AOAC Method[2] Number in 13th Ed.	AOAC Method[2] Number in 16th Ed.	Name/Description
131.110	Milk	Milk solids nonfat ≥ 8 1/4%	16.032	925.23A	Total solids, by hot air oven
		Milkfat ≥ 3 1/4%	16.059	905.02	Roese–Gottlieb
		Vitamin A (if added) ≥ 2000 IU³/qt[4]			
		Vitamin D (if added)—400 IU³/qt[4]	43.195–43.208	936.14	Bioassay line test with rats
131.125	Nonfat dry milk	Moisture ≤ 5% by wt.	16.192	927.05	Vacuum oven
		Milkfat ≤ 1 1/2% by wt.	16.199–16.200	932.06A, 932.06B	Roese–Gottlieb
133.113	Cheddar cheese	Milkfat ≥ 50% by wt. of solids	16.255	933.05	Digest with HCl, Roese–Gottlieb
		Moisture ≤ 39% by wt.	16.233	926.08	Vacuum oven
		Phosphatase level ≤ 3 µg phenol equivalent/ 0.25 g[5]	16.275–16.277	946.03–946.03C	Residual phosphatase
135.110	Ice cream and frozen custard	Total solids ≥ 1.6 lb/gal			
		Milkfat ≥ 10%	16.287, 16.059	952.06, 953.08D	Roese–Gottlieb
		Nonfat milk solids ≥ 10%[6]			
137.165	Enriched flour	Moisture ≤ 15%	14.002, 14.003	925.09, 925.09B	Vacuum oven
		Ascorbic acid ≤ 200 ppm (if added as dough conditioner)			
		Ash[7] ≤ (0.35 + 1/20 of the percent of protein, calculated) on dwb[8])	14.006	923.03	Dry ashing
		(Protein)	2.057	955.04C	Kjeldahl, for nitrate-free samples
		Thiamine, 2.9 mg/lb Riboflavin, 1.8 mg/lb Niacin, 24 mg/lb Iron, 20 mg/lb Calcium (if added), 960 mg/lb			
137.230	Corn grits	Crude fiber ≤ 1.2% dwb[8]	14.062, 14.065	945.38A, 945.38D (modified)	Crude fiber
		Fat ≤ 2.25%	14.062, 14.067	945.38A, 945.38F	Ether extraction
		(Moisture)	14.062, 14.063	945.38A, 945.38B	Vacuum oven
145.110	Canned applesauce	Soluble solids ≥ 9%[9]	22.024	932.12	Refractometer
146.185	Pineapple juice	Soluble solids ≥ 10.5°Brix[10]	31.009	932.14A	Hydrometer (Brix spindle)
		Total acidity ≤ 1.35 g/100 ml (as anhydrous citric acid)			Titration with NaOH[11]
		Brix/acid ratio ≥ 12			Calculated[12]
		Insoluble solids ≥ 5 and ≤ 30%			Calculated from volume of sediment[13]
158.170	Frozen peas	Alcohol insoluble solids ≤ 19%			Extract with alcohol solution; dry insoluble material[14]
163.113	Cocoa	Cocoa fat ≤ 22% and ≥ 10%		925.15	Extraction with petroleum ether
164.150	Peanut butter	Fat ≤ 55%	27.006(a)	948.22	Ether extraction with Soxhlet unit

2-1 table

Selected Chemical Composition Requirements of Some Foods with Standards of Identity (continued)

Section in 21 CFR[1]	Food Product	Requirement	AOAC Method[2]		
			Number in 13th Ed.	Number in 16th Ed.	Name/Description
168.20	Glucose syrup	Total solids ≥ 70% mass/mass (m/m)	31.208–31.209	941.14A, 941.14B	Vacuum oven, with diatomaceous earth
		Reducing sugar ≥ 20% m/m (dextrose equivalent, dwb)	31.220(a)	945.66(a)	Lane–Eynon
		Sulfated ash ≤ 1% m/m, dwb	31.216	945.63B	Dry ashing
		Sulfur dioxide ≤ 40 mg/kg	20.106–20.111	962.16A–963.20C	Modified Monier–Williams
169.175	Vanilla extract	Ethyl alcohol ≥ 35% by volume			
		Vanilla constituent ≥ 1 unit[15]/gal			

[1]CFR, Code of Federal Regulations (1997).
[2]*Official Methods of Analysis* of AOAC International.
[3]IU = International Units.
[4]Within limits of good manufacturing practice.
[5]If pasteurized dairy ingredients are used.
[6]Exceptions clarified.
[7]Excluding ash resulting from any added iron or salts of iron or calcium or wheat germ.
[8]dwb = moisture-free or dry weight basis.
[9]Exclusive of the solids of any added optional nutritive carbohydrate sweeteners; expressed as % sucrose. °Brix, with correction for temperature to the equivalent at 20°C.
[10]Exclusive of added sugars, without added water. As determined by refractometer at 20°C uncorrected for acidity and read as °Brix on International Sucrose Scales. Exception stated for juice from concentrate.
[11]Detailed titration method given in 21 CFR, 145.180 (b)(2)(ix).
[12]Calculated from °Brix and total acidity values, as described in 21 CFR 146.185 (b)(2)(ii).
[13]Detailed method given in 21 CFR 146.185 (b)(2)(iv).
[14]Detailed method given in 21 CFR 158.170 (b)(3).
[15]Defined in 21 CFR 169.3(c); requires measurement of moisture content, according to modification of AOAC Method 7.004 and 7.005.

ing the 1938 FD&C Act as amended, which prohibits adulteration and misbranding of food products. Relevant to food analysis and for FDA-regulated foods, the FDA inspects food processing facilities for compliance with GMP regulations and for any mandatory HACCP inspection programs. The FDA monitors appropriate foods for composition and characteristics relevant to the standards of identity, standards of quality, standards of fill, nutrition labeling, and other labeling regulations. It regulates color additives and the use of food additives for all foods.

Working with the National Marine Fisheries Service to ensure seafood safety, the FDA sets and enforces allowable levels of contaminants and pathogenic microorganisms in seafood. The FDA has jurisdiction over some alcoholic beverages and cooking wines, and handles questions of deleterious substances in alcoholic beverages. Regulations on tolerance levels of pesticide residues in foods and agricultural commodities set by the Environmental Protection Agency are enforced by the FDA. Imported food products regulated by the FDA are subject to inspection upon entry through

United States Customs, and products must comply with United States laws and regulations. The FDA works with individual states and the United States Department of Agriculture to ensure the safety and wholesomeness of dairy products. Also, the FDA has regulatory power over shellfish sanitation for products shipped interstate.

When violations of the FD&C Act are discovered by the FDA through periodic inspections of facilities and products and through analysis of samples, the FDA can use warning letters, seizures, injunctions, or recalls, depending on the circumstances. The FDA cannot file criminal charges, but rather recommends to the Justice Department that court action be taken that might result in fines or imprisonment of offenders. Details of these enforcement activities of the FDA are given in references (1–3).

2.2.2 United States Department of Agriculture

The USDA, created in 1862, is now one of the cabinet level federal agencies within the executive branch of

the United States government. The USDA administers several federal statutes relevant to food composition and analysis; some programs are mandatory and others voluntary.

2.2.2.1 Mandatory Inspection Programs for Fresh and Processed Food Commodities: Standards and Composition

2.2.2.1.1 Meat and Poultry The Meat and Poultry Inspection Program (MPIP) administered by the USDA provides for inspection of the slaughter of certain domestic livestock and poultry and the processing of meat and poultry products. Such inspection applies to all meat and poultry products in interstate or foreign commerce, to prevent the sale and distribution of adulterated or misbranded products. The MPIP reviews foreign inspection systems and packing plants that export meat and poultry to the United States. Imported products are reinspected at ports of entry.

The MPIP derives its authority from the Federal Meat Inspection Act of 1906, updated in 1967; the Poultry Products Inspection Act of 1957; the Agricultural Marketing Act of 1946; the Humane Slaughter Act of 1958; and the Imported Meat Act (a part of the 1930 Tariff Act).

The regulations relating to the inspection and certification of meat and poultry products are published in Title 9 of the CFR. Comprehensive inspection manuals, such as the *Meat and Poultry Inspection Manual* (11), have been developed to assist MPIP and industry personnel to interpret and utilize the regulations. Standards of identity have been established by the Food Safety and Inspection Service (FSIS) of the USDA for many meat products (9 CFR 319), commonly specifying percentages of meat, fat, and water. Analyses are to be conducted using AOAC methods, if available.

The FSIS of the USDA announced in 1996 the implementation of new rules for improving the safety of meat and poultry. A major component of the rules is a HACCP system to be required of all slaughter and processing plants. Phasing in of the HACCP requirement is based on size of the establishment, with very small plants having the latest date for required implementation of January 25, 2000.

2.2.2.1.2 Grains The Federal Grain Inspection Program Service, a program of the Grain Inspection, Packers and Stockyard Administration (GIPSA), within the USDA administers the mandatory requirements of the U.S. Grain Standards Act of 1976 as amended. The regulations to enforce this act and provide for a national inspection system for grain are published in 7 CFR 800. Mandatory official grade standard exist for a number of grains, including barley, oats, wheat, corn, rye, flaxseed, sorghum, soybeans, and triticale. GIPSA has issued many handbooks and instruc-

tions for its inspectors, such as the *Grain Inspection Handbook—Book II* (12). Grades are determined by factors such as test weight per bushel and percentages of heat-damaged kernels, broken kernels, and foreign material. A grade limit is commonly set for moisture, which is as specified by contract or load order grade.

2.2.2.2 Voluntary Grading and Inspection Programs for Fresh and Processed Food Commodities: Standards and Composition

2.2.2.2.1 General Information on Grade Standards Although grade standards developed for foods by the USDA are not mandatory requirements, they are widely used, voluntarily, by food processors and distributors as an aid in wholesale trading, because the quality of a product affects its price. The USDA has issued grade standards for more than 300 food products under authority of the Agricultural Marketing Act of 1946 and related statutes. Grade standards exist for many types of meats, poultry, dairy products, fruits, vegetables, and grains, along with eggs, domestic rabbits, certain preserves, dry beans, rice, and peas. Additional information about each of these is given in the next several sections, except for dairy products, which is given in section 2.3. While complete information regarding the standards was published previously in the CFR, currently only some standards are published in the CFR because they are USDA Agricultural Marketing Service (AMS) Administrative Orders. All grade standards are available as pamphlets from USDA and also are accessible on the Internet.

The Science and Technology Division, AMS, USDA, has analytical laboratories that perform analyses related to food grade standards and general quality control. These analytical services are available for a fee to governmental agencies, outside companies, and individuals. Laboratory tests available include proximate, lipid-related, food additive, chemical and physical component, microbiological, and aflatoxin analyses (7 CFR 91.37). These analyses can help ensure that food products meet grade standard specifications.

Grade standards, issued by the AMS of the USDA for agricultural products and by the Department of Commerce for fishery products, must not be confused with standards of quality set by the FDA or standards of identity set by the FDA or FSIS of the USDA, as discussed previously. A standard of identity establishes or defines what a given food product is; it establishes for some foods which ingredients they must contain. Standards of quality are the minimum standards for some canned fruits and vegetables. Standards for grades may classify products in a range from substandard to excellent in quality. Standards for grades are not required to be stated on the label, but if they are stated, the product must comply with the specifications of the declared grade. Official USDA grading ser-

vices are provided, for a fee, to pickers, processors, distributors, and others who seek official certification of the grades of their products. Such grade standards often are used as quality control tools. Consumers are familiar generally with grade standards for beef, butter, and eggs, but buyers for the retail market utilize grade standards for a wide variety of foods. Major users of standards include institutions such as schools, hospitals, restaurants, prisons, and the Department of Defense (see also section 2.5).

2.2.2.2.2 Fruits and Vegetables The USDA is responsible for ensuring the quality of fruits, vegetables, and related products sold in the United States. The Fresh Products Branch and the Processed Products Branch of the Fruit and Vegetable (FV) Program of the USDA standardize, grade, and inspect fruits and vegetables under various voluntary programs. The standards promulgated for some fresh fruits and vegetables are given in 7 CFR 51 and those for some processed fruits and vegetables are given in 7 CFR 52. Standards for certain other fresh and processed fruits and vegetables are available as AMS pamphlets and on the Internet. Standards for grades of processed fruits and vegetables often include factors such as color, texture or consistency, defects, size and shape, tenderness, maturity, flavor, and a variety of chemical characteristics. Sampling procedures and methods of analysis are commonly given. As an example, partial information about the standards for grades of frozen concentrated orange juice (13) is given in Table 2-2.

A new quality assurance inspection service, based on HACCP, is in the pilot phase and is being offered to the fruit and vegetable industry. The voluntary program is fee-based and is designed to facilitate marketing. The HACCP concept is considered to be a more scientific, analytical, economical approach to food wholesomeness than traditional inspection systems. Once a HACCP plan has been reviewed and accepted by the FV Program, an objective third-party validation audit and series of systems audits are conducted to determine the applicant's effective adherence to the plan. Companies in good standing under this service can use a new official mark for recognition by consumers that they are in the program.

2.2.2.2.3 Meat and Poultry The Livestock and Seed Program of the USDA provides voluntary grading and certification services, as described in 7 CFR 53 (Livestock) and 54 (Meat, Prepared Meats, and Meat Products). The Poultry Program of the USDA provides voluntary inspection and grading services for egg products (7 CFR 55), voluntary grading of shell eggs (7 CFR 56), voluntary grading of poultry products and rabbit products (7 CFR 70), voluntary inspection of poultry (9 CFR 362), and voluntary inspection of rabbits and their edible products (9 CFR 354).

The voluntary inspection and grading program for egg products covers services such as laboratory analyses required but not covered by the mandatory inspection program that exist for eggs and egg products (7 CFR 59) under the Egg Products Inspection Act. Similarly, the voluntary poultry inspection program exists for poultry products not covered by the mandatory regulations of the Poultry Products Inspection Act (9 CFR 381).

2.2.2.2.4 Other Agricultural Commodities GIPSA implements voluntary regulations and standards for inspection and certification of certain agricultural commodities and their products. Such regulations and standards for rough, brown, and milled rice are given in 7 CFR 868. Standards for beans, peas, and lentils are given in GIPSA publications. Grade standards for these products are commonly determined by factors such as defects, presence of foreign material, and insect infestation. The standard for beans also specifies that beans with more than 18% moisture are graded as "high moisture." The regulations state that moisture is to be determined by the use of equipment and procedures prescribed by the GIPSA, or by any method that gives equivalent results. Laboratory test services are made available (Table 2-3), at a fee, for these agricultural commodities, as they are for other food products through inspection and grading programs.

2.2.3 United States Department of Commerce

2.2.3.1 Seafood Inspection Service

The National Oceanic and Atmospheric Administration (NOAA) and the National Marine Fisheries Service (NMFS) are agencies under the United States Department of Commerce that have provided a seafood inspection service since the administration's Reorganization No. 4 of 1970 moved the service from Department of Interior to Commerce. The NOAA Seafood Inspection Program ensures the safety and quality of seafoods consumed in the United States and certified for export through voluntary grading, standardization, and inspection programs, as described in 50 CFR 260-267. NOAA Handbook 25 is comprehensive manual on these subjects entitled *Fishery Products Inspection Manual* (14). The U.S. Standards for Grades of Fishery Products are intended to help the fishing industry maintain and improve quality and to thereby increase consumer confidence in seafoods. Standards are based on attributes such as color, size, texture, flavor, odor, workmanship defects, and consistency.

2.2.3.2 HACCP-Based Program

The NOAA Seafood Inspection Program has an expanding voluntary HACCP-based program available to all aspects of the fishery products industry. Ini-

2-2
table   USDA Standards for Grades of Frozen Concentrated Orange Juice

	QUALITY:[1,2]	
Factors	Grade A	Grade B
Appearance	Fresh orange juice	Fresh orange juice
Reconstitution	Reconstitutes properly	Reconstitutes properly
Color	Very good (Equal to or better than USDA OJ 5)	Good (Not as good as USDA OJ 5, but not off color)
Score points	36–40	32–35
Defects	Practically free	Reasonably free
Score points	18–20	16–17
Flavor	Very good	Good
Score points	36–40	32–35
Total score points	Minimum, 90	Minimum, 80

	ANALYTICAL:[2]							
	Unsweetened		Sweetened		Unsweetened		Sweetened	
Concentrate:								
Brix value: Minimum	41.8[2]		42.0[2]		41.8[2]		42.0[2]	
Brix value/acid ratio:	Min	Max	Min	Max	Min	Max	Min	Max
California/Arizona	11.5:1	19.5:1	12.0:1	19.5:1	10.0:1		10.0:1	
Outside California/Arizona	12.5:1	19.5:1	13.0:1	19.5:1	10.0:1		10.0:1	
Reconstituted juice:								
Brix: Minimum	11.8[2]				11.8[2]			
Soluble orange solids, exclusive of sweetener (percent by weight of finished product): Minimum			11.8				11.8	
Recoverable oil (percent by volume): Maximum	0.035				0.040			

Adapted from (13).
[1]Reconstituted prior to grading.
[2]Terms describing quality and analytical characteristics are defined in the standards, and in some cases AOAC methods are specified.

tially established in 1992 for processors, participants now also include fishing vessels, food service facilities, and retail establishments. The NOAA HACCP-based program includes wholesomeness, labeling and quality factors, in addition to control of the basic food safety hazards, as required by 21 CFR 123. NOAA technical experts also offer consultative services to any in the industry requesting assistance to meet the requirements of the mandatory FDA HACCP regulation. Specialized training beyond HACCP principles and implementation now also includes auditing and sensory workshops.

2.2.3.3 Interaction with FDA and EPA

The FDA and the EPA work with the NMFS for the assurance of seafood safety. The FDA, under the FD&C Act, is responsible for ensuring that seafood shipped or received in interstate commerce is safe, wholesome, and not misbranded or deceptively packaged. The FDA has primary authority in setting and enforcing allowable levels of contaminants and pathogenic

microorganisms in seafood. The EPA assists the FDA in identifying the range of chemical contaminants that pose a human health risk and are most likely to accumulate in seafood. A tolerance of 2.0 parts per million (ppm) for total polychlorinated biphenyls (PCBs) (21 CFR 109.30) is the only formal tolerance specified by the FDA to mitigate human health impacts in seafood. However, the EPA has established tolerances for certain pesticide residues and the FDA has established guidance levels for the toxic elements arsenic, cadmium, chromium, lead, and nickel (15).

2.2.4 United States Bureau of Alcohol, Tobacco, and Firearms

2.2.4.1 Regulatory Responsibility for Alcoholic Beverages

Beer, wines, liquors, and other alcoholic beverages are termed "food" according to the FD&C Act of 1938. However, regulatory control over their quality, standards, manufacture, and other related aspects is spec-

2-3 table Laboratory Test Services Available Through Federal Grain Inspection Service

1. Alpha monoglycerides	35. Moisture and volatile matter
2. Aflatoxin test (other than TLC or minicolumn method)	36. Performance test (prepared bakery mix)
3. Aflatoxin (TLC)	37. Peroxide value
4. Aflatoxin (minicolumn method)	38. Phosphorus
5. Appearance and color	39. Popcorn kernels (total defects)
6. Ash	40. Popcorn ratio/value popcorn
7. Bacteria count	41. Potassium bromate
8. Baking test (cookies)	42. Protein
9. Bostwick (cooked)	43. Rope spore count
10. Bostwick (uncooked/cook test/dispersibility)	44. *Salmonella*
11. Brix	45. Salt or sodium content
12. Calcium	46. Sanitation (filth light)
13. Carotenoid color	47. Sieve test
14. Cold test (oil)	48. Smoke point
15. Color test (syrups)	49. Solid fat index
16. Cooking test (other than corn soy blend)	50. Specific volume (bread)
17. Crude fat	51. *Staphylococcus aureus*
18. Crude fiber	52. Texture
19. Dough handling (baking)	53. Tilletia controversia kuhn (TCK) (Qualitative)
20. *E. coli*	54. Tilletia controversa kuhn (TCK) (Quantitative)
21. Falling number	55. Unsaponifiable matter
22. Fat (acid hydrolysis)	56. Urease activity
23. Fat stability (AOM)	57. Visual exam (hops pellet)
24. Flash point (open and close up)	58. Visual exam (insoluble impurities, oils, and shortening)
25. Free fatty acid	59. Visual exam (pasta)
26. Hydrogen ion activity (pH)	60. Visual exam (processed grain products)
27. Iron enrichment	61. Visual exam (total foreign material other than cereal grains)
28. Iodine number/value	62. Vitamin enrichment
29. Linolenic acid (fatty acid profile)	63. Vomitoxin (TLC)
30. Lipid phosphorus	64. Vomitoxin (Qualitative)
31. Lovibond color	65. Vomitoxin (Quantitative)
32. Margarine (nonfat solids)	66. Water activity
33. Moisture	67. Wiley melting point
34. Moisture average (crackers)	68. Other laboratory tests

From 7 CFR 868.90–868.91 (1997).

ified by the Federal Alcohol Administration Act, which is enforced by the Bureau of Alcohol, Tobacco, and Firearms of the U.S. Department of the Treasury. Issues regarding the composition and labeling of most alcoholic beverages are handled by the Bureau. However, the FDA has jurisdiction over certain other alcoholic beverages and cooking wines. The FDA also deals with questions of sanitation, filth, and the presence of deleterious substances in alcoholic beverages.

2.2.4.2 Standards and Composition of Beer, Wine, and Distilled Beverage Spirits

Information related to definitions, standards of identity, and certain labeling requirements for beer, wine, and distilled beverage spirits is given in 27 CFR 1-296. Standards of identity for these types of beverages stipulate the need for analyses such as percent alcohol by volume, total solids content, volatile acidity, and calculated. For example, the fruit juice used for the production of wine is often specified by its °Brix and total solids content. The maximum volatile acidity (calculated as

acetic acid and exclusive of sulfur dioxide for grape wine must not be more than 0.14 g/100 ml (20°C) for natural red wine and 0.12 g/ 100 ml for other grape wine (27 CFR 4.21). The percent alcohol by volume is often used as the criterion for class or type designation of alcoholic beverages. For example, dessert wine is grape wine with an alcoholic content in excess of 14% but not in excess of 24% by volume, while table wines have an alcoholic content not in excess of 14% alcohol by volume (27 CFR 4.21). No product with less than 0.5% alcohol by volume is permitted to be labeled "beer," "lager beer," "lager," "ale," "porter," "stout," or any other class or type designation normally used for malt beverages with higher alcoholic content (27 CFR 7.24).

2.2.5 United States Environmental Protection Agency

The EPA was established as an independent agency in 1970 through a reorganization plan to consolidate cer-

tain federal government environmental activities. The EPA regulatory activities most relevant to this book are control of pesticide residues in foods, drinking water safety, and the composition of effluent from food processing plants.

2.2.5.1 Pesticide Registration and Tolerance Levels

Pesticides are chemicals intended to protect our food supply by controlling harmful insects, diseases, rodents, weeds, bacteria, and other pests. However, most pesticide chemicals can have harmful effects on people, animals, and the environment if they are improperly used. The three federal laws relevant to protection of food from pesticide residues are certain provisions of the Federal FD&C Act, the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), as amended, and the Food Quality Protection Act of 1996. FIFRA, supplemented by the FD&C Act, authorizes a comprehensive program to regulate the manufacturing, distribution, and use of pesticides, along with a research effort to determine the effects of pesticides.

The Food Quality Protection Act amends both the FD&C Act and FIFRA, to take pesticides out of the section of the FD&C Act that includes the Delaney Clause. This was done by changing the definition of a "food additive" to exclude pesticides. This redefinition leaves the Delaney Clause greatly reduced in scope and thus less relevant.

The EPA registers approved pesticides and sets tolerances for pesticide residues. Both areas of responsibility are described in Chapter 20, section 20.1.1.1, and the setting of tolerance levels is described here. The EPA is authorized to establish an allowable limit or tolerance for any detectable pesticide residues that might remain in or on a harvested food or feed crop. The tolerance level is often many times less than the level expected to produce undesirable health effects in humans or animals. Tolerances are established based on factors that include registration data, consumption pattern, age groups, mode of action, chemistry of the compound, toxicological data, plant and animal physiology, efficacy data, and risk assessment. While the EPA establishes the tolerance levels, the FDA enforces the regulations by collecting and analyzing food samples, mostly agricultural commodities. Livestock and poultry samples are collected and analyzed by the USDA. Pesticide residue levels that exceed the established tolerances are considered in violation of the FD&C Act.

The Food Quality Protection Act of 1996 requires an explicit determination that tolerances are safe for children, and consideration of children's special sensitivity and exposure to pesticide chemicals. It includes

an additional safety factor of up to 10-fold, if necessary, to account for uncertainty in data relative to children. The 1996 law requires that all existing tolerances be reviewed within 10 years to make sure they meet the requirements of new health-based safety standards established by law.

Regulations regarding pesticide tolerances in raw agricultural chemicals are given in 40 CFR 180, and for processed foods in 40 CFR 185. The 40 CFR 180 specifies general categories of products and specific commodities with tolerances or exemptions, and in some cases which part of the agricultural product is to be examined. Agricultural products covered include a wide variety of both plants (e.g., fruits, vegetables, grains, legumes, nuts) and animals (e.g., poultry, cattle, hogs, goats, sheep, horses, eggs, milk). Unless otherwise noted, the specific tolerances established for the pesticide chemical apply to residues resulting from their application prior to harvest or slaughter. Tolerances are expressed in terms of parts by weight of the pesticide chemical per 1 million parts by weight of the raw agricultural commodity (i.e., ppm). For example, the residue tolerance for the pesticide chloropyrifos ranges from 0.01 to 13 ppm, depending on the commodity (40 CFR 180.342) (Table 2-4). Tolerance levels for selected pesticides and insecticides permitted in foods as food additives are given in Table 2-5.

The analytical methods to be used for determining whether pesticide residues are in compliance with the tolerance established are identified among the methods contained or referenced in the *Pesticide Analytical Manual* (16) maintained by and available from the

2-4

table Tolerances for Residues of Chloropyrifos on Selected Commodities[1]

Commodity	Parts per Million
Alfalfa hay	13
Bananas, whole	0.1
Broccoli	1
Cabbage	1
Cattle, fat	0.3
Cattle, meat	0.05
Cattle, MBYP[2]	0.05
Corn, field, grain	0.05
Corn, forage	8
Eggs	0.01
Milkfat	0.25
Milk, whole	0.01
Supote (California)	0.05(R)[3]
Strawberries	0.2
Sugarcane	0.01

From 40 CFR 150.342 (1997).
[1]Chloropyrifos is also known as Dunsban™ and Lorsban™.
[2]MBYP = meat by-products
[3]R = regional tolerance.

2-5 table Tolerance for Selected Insecticides (I), Fungicides (F), and Herbicides (H) Classified as Food Additives Permitted in Foods for Human Consumption

Section	Food Additive	Chemical Classification	Food	Tolerance
180.350	Benomyl (F)	Carbamate	Apple pomace, dried	70
			Citrus pulp, dried	50
			Grape pomace, dried	125
			Raisin waste	125
			Raisins	50
			Rice hulls	20
			Tomato products, conc.	50
180.1050	Chlorpyrifos-methyl (I)	Organophosphate	Barley, milling fractions	90
			Oats, milling fractions[1]	130
			Rice, milling fractions[1]	30
			Sorghum, milling fractions[1]	90
			Wheat, milling fractions[1]	30
180.1580	Deltamethrin (I)	Pyrethroid	Tomato products, conc.	1.0
185.4850	Picloram (H)	Chloropyridine carboxylic acid	Barley, flour	1.0
			Barley, milled fraction	3.0
			Oats, milled fraction[1]	3.0
			Wheat, flour	1.0
			Wheat, milled/fraction[1]	3.0

Adapted from 40 CFR 185 (1997).
[1]Except flour.

FDA. The EPA also publishes methods books for pesticides (17, 18). The methods must be sensitive and reliable at and above the tolerance level. Pesticides are generally detected and quantitated by gas chromatographic or high performance liquid chromatographic methods (see Chapters 20, 32, and 33).

2.2.5.2 Drinking Water Standards and Contaminants

The EPA administers the Safe Drinking Water Act of 1974, which is to provide for the safety of drinking water supplies in the United States and to enforce national drinking water standards. The EPA has identified potential contaminants of concern and established their maximum acceptable levels in drinking water. The EPA has primary responsibility to establish the standards, while the states enforce them and otherwise supervise public water supply systems and sources of drinking water. Primary and secondary drinking water regulations have been established; enforcement of the former is mandatory, whereas enforcement of the latter is optional. The national primary and secondary drinking water regulations are given in 40 CFR 141 and 143, respectively. Recently, concerns have been expressed regarding the special standardization of water used in the manufacturing of foods and beverages.

Maximum contaminant levels (MCL) for primary drinking water are set for certain inorganic and organic chemicals, turbidity, certain types of radioac-

tivity, and microorganisms. Sampling procedures and analytical methods for the analysis of chemical contaminants are specified, with common reference to *Standard Methods for the Examination of Water and Wastewater* (19) published by the American Public Health Association; *Methods of Chemical Analysis of Water and Wastes* (20), published by the EPA; and *Annual Book of ASTM Standards* (21), published by the American Society for Testing Materials. Methods commonly specified for the analysis of inorganic contaminants in water include atomic absorption (direct aspiration or furnace technique), inductively coupled plasma (see Chapter 28), ion chromatography (see Chapter 32), and ion selective electrode (see Chapter 10) (Table 2-6).

2.2.5.3 Effluent Composition from Food Processing Plants

In administering the Federal Water Pollution and Control Act, the EPA has developed effluent guidelines and standards that cover various types of food processing plants. Regulations promulgated under 40 CFR 403–471 prescribe effluent limitation guidelines for existing sources, standards of performance for new sources, and pretreatment standards for new and existing sources. Point sources of discharge of pollution are required to comply with these regulations, where applicable. Regulations are prescribed for specific foods under the appropriate point source category: dairy products processing (40 CFR 405), grain mills (40 CFR

2-6 table	Detection Limits for Inorganic Contaminants in Drinking Water

Contaminant	MCL[1] (mg/liter)	Analytical Method	Detection Limit (mg/liter)
Antimony	0.006	Atomic absorption; furnace technique	0.003
		Atomic absorption; platform	0.0008[2]
		ICP–mass spectrometry	0.0004
		Hydride–atomic absorption	0.001
Asbestos	7 MFL[3]	Transmission electron microscopy	0.01 MFL
Barium	2	Atomic absorption; furnace technique	0.002
		Atomic absorption; direct aspiration	0.1
		Inductively coupled plasma	0.002(0.001)
Beryllium	0.004	Atomic absorption; furnace technique	0.0002
		Atomic absorption; platform	0.00002[2]
		Inductively coupled plasma[4]	0.0003
		ICP–mass spectrometry	0.0003
Cadmium	0.005	Atomic absorption; furnace technique	0.0001
		Inductively coupled plasma	0.001
Chromium	0.1	Atomic absorption; furnace technique	0.001
		Inductively coupled plasma	0.007(0.001)
Cyanide	0.2	Distillation, spectrophotometric[5]	0.02
		Distillation, automated, spectrophotometric[5]	0.005
		Distillation, selective electrode[5]	0.05
		Distillation, amenable, spectrophotometric[6]	0.02
Mercury	0.002	Manual cord vapor technique	0.0002
		Automated cold vapor technique	0.0002
Nickel		Atomic absorption; furnace technique	0.001
		Atomic absorption; platform	0.0006[2]
		Inductively coupled plasma[4]	0.005
		ICP–mass spectrometry	0.0005
Nitrate	10 (as N)	Manual cadmium reduction	0.01
		Automated hydrazine reduction	0.01
		Automated cadmium reduction	0.05
		Ion selective electrode	1
		Ion chromatography	0.01
Nitrite	1 (as N)	Spectrophotometric technique	0.01
		Automated cadmium reduction	0.05
		Manual cadmium reduction	0.01
		Ion chromatography	0.004
Selenium	0.05	Atomic absorption; furnace	0.002
		Atomic absorption; gaseous hydride	0.002
Thallium	0.002	Atomic absorption; furnace	0.001
		Atomic absorption; platform	0.0007[2]
		ICP–mass spectrometry	0.0003

From 40 CFR 141.23 (1997).
[1] MCL = maximum contaminant level; maximum permissible level of a contaminant in water as specified in 40 CFR 141.2 (1996).
[2] Lower MDLs are reported using stabilized temperature graphite furnace atomic absorption.
[3] MFL = million fibers per liter > 10 μm.
[4] Using a 2X preconcentration step as noted in Method 200.7. Lower MDLs may be achieved when using a 4X preconcentration.
[5] Screening method for total cyanides.
[6] Measures "free" cyanides.

406), canned and preserved fruits and vegetables processing (40 CFR 407), canned and preserved seafood processing (40 CFR 408), sugar processing (40 CFR 409), and meat products (40 CFR 432). Effluent characteristics commonly prescribed for food processing plants are biochemical oxygen demand (BOD) (see Chapter 24), total soluble solids (TSS) (see Chapter 5), and pH (see Chapter 7), as shown in Table 2-7 for efflu-

ent from a plant that makes natural and processed cheese. The test procedures for measurement of effluent characteristics are prescribed in 40 CFR 136.

2.2.6 United States Customs Service

Over 100 countries export food, beverages, and related edible products to the United States. The U.S. Customs

2-7
table Effluent Limitations for Plants Processing Natural and Processed Cheese

Effluent Limitations	Effluent Characteristics					
	Metric Units[1]			English Units[4]		
	BOD5[2]	TSS[3]	pH	BOD5	TSS	pH
Processing more than 1,000,000 lb/day of milk equivalent						
Maximum for any 1 day	0.716	1.088	([5])	0.073	0.109	([5])
Avg of daily values for 30 consecutive days shall not exceed	0.290	0.435	([5])	0.029	0.044	([5])
Processing less than 100,000 lb/day of milk equivalent						
Maximum for any 1 day	0.976	1.462	([5])	0.098	0.146	([5])
Avg of daily values for 30 consecutive days shall not exceed	0.488	0.731	([5])	0.049	0.073	([5])

Adapted from 40 CFR 405.62 (1997).
[1]Kilograms per 1000 lbs of BOD5 input.
[2]BOD5 refers to biochemical oxygen demand measurement after 5 days of incubation.
[3]TSS refers to total soluble solids.
[4]Pounds per 100 lbs of BOD5 input.
[5]Within the range 6.0 to 9.0.

Service (USCS) assumes the central role in ensuring that imported products are taxed properly, safe for human consumption, and not economically deceptive. The USCS receives assistance from the FDA and USDA as it assumes these responsibilities. The major regulations promulgated by the USCS are given in Title 19 of the CFR.

2.2.6.1 Harmonized Tariff Schedule of the United States (TSUSA)

All goods imported into the United States are subject to duty or duty-free entry according to their classification under applicable items in the Harmonized Tariff Schedule of the United States (TSUSA). The TSUSA can be purchased annotated looseleaf edition from the U.S. Government Printing Office (22). The United States tariff system has official tariff schedules for over 400 edible items exported into the United States. The TSUSA specifies the food product in detail and gives the general rate of duty applicable to that product coming from most countries and any special higher or lower rates of duty for certain other countries.

2.2.6.2 Food Composition and the TSUSA

The rate of duty for certain food products is determined by their chemical composition. The rate of duty on, some dairy products is determined in part by the, fat content, as shown for milk and cream in Table 2-8. The tariff for some syrups is determined by the fructose content, for some chocolate products by the sugar or butterfat content, for butter substitutes by the butterfat content, and for some wines by their alcohol content (percent by volume).

2.2.7 United States Federal Trade Commission

The Federal Trade Commission (FTC) is the most influential of the federal agencies that have authority over various aspects of advertising and sales promotion practices for foods in the United States. The major role of the FTC is to keep business and trade competition free and fair.

2.2.7.1 Enforcement Authority

The Federal Trade Commission Act of 1914 authorizes the FTC to protect both the consumer and the business person from anticompetitive behavior and unfair or deceptive business and trade practices. The FTC periodically issues industry guides and trade regulations and rules that tell businesses what they can and cannot do. These issuances are supplemented with advisory opinions given to corporations and individuals upon request. The proposal of any new rules, guidelines, or regulations is preceded by widespread notice or announcement in the Federal Register and comments are invited. The FTC not only has guidance and preventive functions but is also authorized to issue complaints or shutdown orders and sue for civil penalties for violation of trade regulation rules. The Bureau of Consumer Protection is one of the FTC bureaus that enforce and develop trade regulation rules.

2.2.7.2 Food Labels, Food Composition, and Deceptive Advertising

While the Fair Packaging and Labeling Act of 1966 is administered by the FTC, that agency does not have

2-8 table   U.S. Harmonized Tariff Schedule for Milk and Cream

Article Description	Units of Quantity	Rates of Duty 1 General[1]	Rates of Duty 1 Special[2]	2[3]
Milk and cream, not concentrated or containing added sugar or other sweetening matter:				
Of a fat content, by weight, not exceeding 1%	Liters	0.37¢/liter	Free (CA, E, IL, J, MX)	0.5¢/liter
Of a fat content, by weight, exceeding 1% but not exceeding 6%:				
For not over 11,356,236 liters entered in any calendar year	Liters	0.47¢/liter	Free (Ca, E, IL, J, MX) 0.3¢/liter (CA)	1.7¢/liter
Other	Liters	1.7¢/liter	Free (IL,MX) 1¢/liter (CA)	1.7¢/liter
Of a fat content, by weight exceeding 6%:				
Of a fat content, by weight, not exceeding 45%:				
Described in general note 15	Liters	3.2¢/liter	Free (E, IL, J, MX) 0.3¢/liter (CA)	15¢/liter
Described in additional U.S. note 5	Liters	3.2¢/liter	Free (E, IL) 9¢/liter (CA)	15¢/liter
Other	Liters	84¢/liter	See subheading 9906.04.01-9906.03 (MX)	90.8¢/liter

Adapted from (22) (Section 0401).
[1]General tariff rate that applies to most countries.
[2]Special lower tariff treatment, with symbols referring to specific programs.
[3]Special tariff rate applying to certain countries.

specific authority over the packaging and labeling of foods. The FTC and FDA have agreed upon responsibilities: FTC has primary authority over advertising of foods and FDA has primary authority over labeling of foods.

Grading, standards of identity, and labeling of foods regulated by several federal agencies as described previously have eliminated many potential problems in the advertising of foods. Such federal regulations and voluntary programs have reduced the scope of advertising and other forms of product differentiation. Misleading, deceptive advertising is less likely to be an issue and is more easily controlled. For example, foods such as ice cream, mayonnaise, and peanut butter have standards of identity that set minimum ingredient standards. If these standards are not met, the food must be given a different generic designation (e.g., salad dressing instead of mayonnaise) or be labeled "imitation." Grading, standards, and labeling of food aid consumers in making price-quality comparisons. Once again, analyses of chemical composition play an important role in developing and setting these grades, standards, and labels. In many cases in which the FTC intervenes, data from a chemical analysis become central evidence for all parties involved.

2.3 REGULATIONS AND RECOMMENDATIONS FOR MILK

The safety and quality of milk and dairy products in the United States are the responsibility of both federal (FDA and USDA) and state agencies. The FDA has regulatory authority over the dairy industry in interstate commerce, while the USDA involvement with the dairy industry is voluntary and service oriented. Each state has its own regulatory office for the dairy industry within that state. The various regulations for milk involve several types of chemical analyses.

2.3.1 FDA Responsibilities

The FDA has responsibility under the FD&C Act, the Public Health Service Act, and the Import Milk Act to assure consumers that the United States milk supply and imported dairy products are safe, wholesome, and not economically deceptive. Processors of both Grade A and Grade B milk are required under FDA regulations to take remedial action when conditions exist that could jeopardize the safety and wholesomeness of milk and dairy products being handled. As described in section 2.2.1.2, the FDA also promulgates standards of

identity and labeling, quality, and fill-of-container requirements for milk and dairy products moving in interstate commerce.

For Grade A milk and dairy products, each state shares with the FDA the responsibility of ensuring safety, wholesomeness, and economic integrity. This is done through a Memorandum of Understanding with the National Conference on Interstate Milk Shipments, which is comprised of all 50 states. In cooperation with the states and the dairy industry, the FDA has also developed for state adoption model regulations regarding sanitation and quality aspects of producing and handling Grade A milk. These regulations are contained in the *Grade A Pasteurized Milk Ordinance* (PMO) (23), which all states have adopted as minimum requirements.

The standards for Grade A pasteurized milk and milk products and bulk-shipped heat-treated milk products under the PMO are given in Table 2-9. The PMO specifies that "all sampling procedures and required laboratory examinations shall be in substantial compliance with the ... Edition of *Standard Methods for the Examination of Dairy Products* of the American Public Health Association, and the ... Edition of *Official Methods of Analysis* of the Association of Official Analytical Chemists. (Insert edition number current at time of adoption.)" (23–25).

The FDA monitors state programs for compliance with the PMO and trains state inspectors. To facilitate movement of Grade A milk in interstate commerce, a federal–state certification program exists: the Interstate Milk Shippers (IMS) Program. This program is maintained by the National Conference on Interstate Milk Shipments, which is a voluntary organization that includes representatives from each state, the FDA,

the USDA, and the dairy industry. In this program, the producers of Grade A pasteurized milk are required to pass inspections and be rated by cooperating state agencies, based on PMO sanitary standards, requirements, and procedures. The ratings appear in the IMS List (26), which is published by the FDA, and made available to state authorities and milk buyers to ensure the safety of milk shipped from other states.

2.3.2 USDA Responsibilities

Under authority of the Agricultural Marketing Act of 1946, the Dairy Quality Program of the USDA offers voluntary grading services for manufactured or processed dairy products (7 CFR 58). If USDA inspection of a dairy manufacturing plant shows that good sanitation practices are being followed to meet the requirements in the *General Specifications for Dairy Plants Approved for USDA Inspection and Grading Service* (27), the plant qualifies for the USDA services of grading, sampling, testing, and certification of its products. A product such as nonfat dry milk is graded based on flavor, physical appearance, and various laboratory analyses, the last of which is given in Table 2-10.

As with the USDA voluntary grading programs for other foods described in section 2.2.2.2, the USDA has no regulatory authority regarding dairy plant inspections and cannot require changes in plant operations. The USDA can only decline to provide the grading services, which are available to the dairy plants for a fee. The USDA, under an arrangement with the FDA, assists states in establishing safety and quality regulations for manufacturing-grade milk. Much as described previously for the FDA with Grade A milk, the USDA has developed model regulations for state adoption regarding the quality and sanitation aspects of

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2-9
table

Pasteurized Milk Ordinance Standards for Grade A Pasteurized Milk and Milk Products and Bulk-Shipped Heat-Treated Milk Products

Criteria	Requirement
Temperature	Cooled to 7°C (45°F) or less and maintained thereat
Bacterial limits[1]	20,000 per ml
Coliform	Not to exceed 10 per ml: Provided that, in the case of bulk milk transport tank shipments, shall not exceed 100 per ml
Phosphatase[2]	Less than 1 μg per ml by the Scharer Rapid Method
Drugs[3]	No positive results on drug residue detection methods

Adapted from (23).
[1]Not applicable to cultured products.
[2]Not applicable to bulk-shipped heat-treated milk products.
[3]Reference to specific laboratory techniques.

2-10
table

U.S. Standards for Grades of Nonfat Dry Milk (Spray Process): Classification According to Laboratory Analysis

Laboratory Tests[1]	U.S. Extra Grade	U.S. Standard Grade
Bacterial estimate, standard plate count per gram	40,000	75,000
Milkfat content, percent	1.25	1.50
Moisture content, percent	4.0	5.0
Scorched particle content, mg	15.0	22.5
Solubility index, ml	1.2	2.0
U.S. High Heat	2.0	2.5
Titratable acidity (lactic acid), percent	0.15	0.17

From 7 CFR 58.2528 (1997).
[1]All values are maximum allowed.

producing and handling manufacturing-grade milk. These regulations are given in the *Milk for Manufacturing Purposes and Its Production and Processing, Recommended Requirements* (28). The states that have Grade B milk have essentially adopted these model regulations.

2.3.3 State Responsibilities

As described previously, individual states have enacted safety and quality regulations for Grade A and manufacturing-grade milk that are essentially identical to those in the PMO and the USDA Recommended Requirements, respectively. The department of health or agriculture in each state normally is responsible for enforcing these regulations. The states also establish their own standards of identity and labeling requirements for milk and dairy products, which are generally similar to the federal requirements.

2.4 REGULATIONS AND RECOMMENDATIONS FOR SHELLFISH

Shellfish include fresh or frozen oysters, clams, and mussels. They may transmit intestinal diseases such as typhoid fever or act as carriers of natural or chemical toxins. This makes it very important that they be obtained from unpolluted waters and handled and processed in a sanitary manner.

2.4.1 State and Federal Shellfish Sanitation Programs

The growing, handling, and processing of shellfish must comply not only with the general requirements of the FD&C Act but also with the requirements of state health agencies cooperating in the National Shellfish Sanitation Program (NSSP) administered by the FDA (29). The FDA has no regulatory power over shellfish sanitation unless the product is shipped interstate. However, the Public Health Service Act authorizes the FDA to make recommendations and to cooperate with state and local authorities to ensure the safety and wholesomeness of shellfish. Under special agreement, Canada, Japan, Korea, Iceland, Mexico, Australia, England, and New Zealand are in the NSSP and are subject to the same sanitary controls as required in the United States. Through the NSSP, state health personnel continually inspect and survey bacteriological conditions in shellfish-growing areas. Any contaminated location is supervised or patrolled so that shellfish cannot be harvested from the area. State inspectors check harvesting boats and shucking plants before issuing approval certificates, which serve as operating

licenses. The certification number of the approved plant is placed on each shellfish package shipped.

2.4.2 Natural and Environmental Toxic Substances in Shellfish

A major concern is the ability of shellfish to concentrate radioactive material, insecticides, and other chemicals from their environment. Thus, one aspect of the NSSP is to ensure that shellfish-growing areas are free from sewage pollution and toxic industrial waste. Pesticide residues in shellfish are usually quantitated by gas chromatographic techniques, and heavy metals such as mercury are commonly quantitated by atomic absorption spectroscopy (e.g., AOAC Method 977.15). Another safety problem with regard to shellfish is the control of natural toxins, which is a separate issue from sanitation. The naturally occurring toxins are produced by planktonic organisms and testing is conducted using a variety of assays. Control of this toxicity is achieved by a careful survey followed by prohibition of harvesting from locations inhabited by toxic shellfish.

2.5 VOLUNTARY FEDERAL RECOMMENDATIONS AFFECTING FOOD COMPOSITION

2.5.1 Food Specifications, Food Purchase, and Government Agencies

Large amounts of food products are purchased by federal agencies for use in domestic (e.g., school lunch) and foreign programs, prisons, veterans' hospitals, the armed forces, and other organizations. Specifications or descriptions developed for many food products are used by federal agencies in procurement of foods, to ensure the safety and quality of the product specified. Such specifications or descriptions often include information that requires assurance of chemical composition.

2.5.1.1 Federal Specifications

A Federal Specification serves as a document for all federal user agencies to procure essential goods and services on a competitive basis. All such specifications for foods should include at least the following information:

1. Name of product
2. Grade or quality designation
3. Size of container or package
4. Number of purchase units
5. Any other pertinent information

2.5.1.2 Commercial Item Descriptions

Commercial Item Descriptions (CIDs) are a series of federal specifications that usually contain the same basic components, with certain optional elements. CIDs are used in lieu of federal specifications to purchase commercial off-the-shelf products of good commercial quality. These products must adequately serve government requirements and have an established commercial acceptability. The Agricultural Marketing Service of the USDA has management authority for all food federal standardization documents, including CIDs. The basic format of a CID follows:

1. Scope—name of product
2. Classification—type, grade, class
3. Salient characteristics—analytical requirements, procedure, and testing; preparation of sample; test results
4. Regulatory requirements—federal and state mandatory requirements and regulations, where applicable
5. Quality assurance provisions—contractor certification clause, and inspection requirements, when needed
6. Packaging—preservation, packaging, packing, labeling, and case marketing
7. Notes—any special notes

2.5.1.3 Other Specifications

In addition to Federal Specifications and CIDs, federal agencies use other terms for the specifications they use in the purchase of foods. These include Purchase Product Description (PPD), USDA Specifications, Commodity Specifications, and Military Specifications.

2.5.1.4 Examples of Specifications for Food Purchase

Various CIDs, PPDs, Federal Specifications, or USDA Specifications are used by the USDA (Commodity Procurement Branch, Livestock and Seed Program, Agricultural Marketing Service) to purchase meat products for programs such as school lunches. For example, the CID for canned tuna (30) specifies salt/sodium levels, with analysis to be done by the AOAC flame photometric method for sodium and potassium in seafood. The Institutional Meat Purchase Specifications (a USDA specification) for frozen ground pork (31) and frozen ground beef products (32) state maximum allowable fat contents. In specifications for many meat products, the purchaser may specify discount ranges for fat content analysis below the maximum allowable

fat content, such that a premium price is paid for a product with a lower fat content. The Institutional Meat Purchase Specification for lean finely textured beef specifies minimums for protein content, protein efficiency ratio, and essential amino acid content (32).

Commodity Specifications for a variety of poultry products have been issued by the USDA (Commodity Procurement Branch, Poultry Program, Agricultural Marketing Service). Samples for analyses may be submitted to USDA laboratories. Specifications generally state how the USDA laboratory will sample the product and report the results, and in some cases what method will be used to do the assay. For example, the moisture content of dried egg mix (33) will be analyzed in accordance with Laboratory Methods for Egg Products (34). Such a dried egg mix is to consist of liquid whole eggs, nonfat dry milk, vegetable oil, and salt. Cottonseed corn or soybean oil can be used as the vegetable oil, with specifications given for the following, as determined by AOCS test methods: free fatty acid value, peroxide value, linolenic acid, moisture and volatile matter, iodine value, and Lovibond color values (see Chapter 14 for some of these tests).

Commodity Specifications have been developed for bulk dairy products purchased by the Commodity Credit Corporation of the USDA under the Dairy Price Support Program (35). For example, the moisture and vitamin A contents of nonfat dry milk are specified, as are the moisture content of cheese and butter, and the milkfat content and pH of butter. Pasteurized process American cheese (36) and mozzarella cheese (37) "for use in domestic donation programs" have specifications on moisture and milk fat contents.

The Defense Personnel Support Center of the Defense Logistics Agency, Department of Defense, utilizes a variety of specifications, standards, and notes in the purchase of food for the military: USDA Notices or Purchase Specifications (Schedules), CIDs, Military Specifications, and Non Governmental Standards (e.g., Institutional Meat Purchase Specifications). For example, they use CID for syrup (38) and instant tea (39), USDA Specification for slab or sliced bacon (40), USDA Institutional Meat Purchase Specifications for frozen frankfurters (41), and Military Specification for beef stew (dehydrated, cooked) (a combat ration item) (42).

2.5.2 National Conference on Weights and Measures: State Food Packaging Regulations

Consumers assume that the weighing scale for a food product is accurate and that a package of flour, sugar, meat, or ice cream contains the amount claimed on the label. While this assumption is usually correct, city or county offices responsible for weights and measures need to police any unfair practices. Leadership in this

area is provided by the National Conference on Weights and Measures (NCWM) (43).

2.5.2.1 National Conference on Weights and Measures

The NCWM was established in 1905 by the National Institute of Standards and Technology (NIST) (formerly the National Bureau of Standards), which is part of the U.S. Department of Commerce. This came from a need to bring about uniformity in state laws referring to weights and measures and to create close cooperation between the state measurement services and NIST. The NCWM has no regulatory power, but it develops many technical, legal, and general recommendations in the field of weights and measures administration and technology. The NCWM is a membership organization comprised of state and local weights and measures regulatory officers; other officials of federal, state, and local governments; and representatives of manufacturers, industry, business, and consumer organizations. It assembles for an annual meeting of decision making officials and generates uniformity in the regulations issued by these officials concerning weights and measures.

2.5.2.2 NIST Handbook 133

The NIST Handbook 133, *Checking the Net Contents of Packaged Goods* (44), gives model state packaging and labeling regulations that have been adopted by a majority of states. The Handbook provides detailed procedures for (1) testing packages labeled by liquid or dry volume; length, area, count, and combinations of labeled quantities; (2) testing certain hard-to-measure prepackaged goods; and (3) sampling to determine compliance with regulations. The Handbook specifies that the average quantity of contents of packages must at least equal the labeling quantity, with the variation between the individual package net contents and the labeled quantity not too "unreasonably large." Variations are permitted within the bounds of GMPs and are due to gain or loss of moisture (within the bounds of good distribution practice). For certain products (e.g., flour, pasta, rice) this requires careful monitoring of moisture content and control of storage conditions by the manufacturer.

2.6 INTERNATIONAL STANDARDS AND POLICIES

With the need to compete in the worldwide market, employees of food companies must be aware that allowed food ingredients, names of food ingredients, required and allowed label information, and standards for foods and food ingredients differ between countries. For example, colorings and preservatives allowed in foods differ widely between countries, and nutritional labeling is not universally required. To develop foods for, and market foods in, a global economy, one must seek such information from international organizations and from organizations in specific regions and countries.

2.6.1 Codex Alimentarius

The Codex Alimentarius Commission (Alimentarius is Latin for "code concerned with nourishment") was established in 1962 by two United Nations organizations, the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), to develop international standards and safety practices for foods and agricultural products (45–47). The standards, published in the Codex Alimentarius, are intended to protect consumers' health, ensure fair business practices in food trade, and facilitate international trade of foods (46).

The *Codex Alimentarius* is published in 13 volumes: one on general requirements (includes labeling, food additives, contaminants, irradiated foods, import/export inspection, and food hygiene), nine on standards and codes of practice compiled on a commodity basis, two on residues of pesticides and veterinary drugs in foods, and one on methods of analysis and sampling (Table 2-11). Codex has efforts to validate and harmonize methods of food safety analysis among countries and regions, to help maintain the smooth flow of international commerce, and ensure appropriate decisions on food exports and imports. Codex has adopted the HACCP concept as the preferred means to ensure the safety of perishable foods, and is determining how HACCP will be implemented in *Codex Alimentarius*.

Recently Codex has strengthened it commitment to base food standards on strong science, rather than on social or cultural factors, economics, or trade policies. The setting of international standards on food quality by Codex has been a high priority in world trade to minimize "nontariff" trade barriers. International trade of food and raw agricultural products has increased due to reduced economic trade restrictions and tariffs imposed, but food standards set in the past by some countries created nontariff trade barriers. Food standards developed by Codex are intended to overcome the misuse of standards by a country, when the standards do more to protect products in a country from the competition of imports than to protect the health of consumers.

2-11
table

Content of the *Codex Alimentaris* (46)

Volume	Subject
1A	General Requirements
1B	General Requirements (Food Hygiene)
2A	Pesticide Residues in Foods (General Text)
2B	Pesticide Residues in Foods (Maximum Residue Limits)
3	Residues in Veterinary Drugs in Foods
4	Foods for Special Dietary Uses
5A	Processed and Quick-Frozen Fruits and Vegetables
5B	Fresh Fruits and Vegetables
6	Fruit Juices
7	Cereals, Pulses (Legumes) & Derived Products and Vegetable Proteins
8	Fats and Oils and Related Products
9	Fish and Fishery Products
10	Meat and Meat Products, Soups and Broths
11	Sugars, Cocoa Products and Chocolate, and Misc. Products
12	Milk and Milk Products
13	Methods of Analysis and Sampling

Decisions at the 1994 Uruguay Round of the General Agreement on Tariffs and Trade (GATT) strengthened the role of Codex as the principal standard-setting group internationally for the quality and safety of foods. The United States is among the 156 countries that are members of Codex. The United States recognizes treaty obligations related to Codex that have arisen from GATT. As a result, representatives of the FDA, USDA, and EPA (the three United States federal agencies that participate in Codex) in 1995 developed a strategic plan for Codex that included greater United States acceptance of Codex standards. In the United States, there is increased participation of nongovernmental organizations (e.g., Grocery Manufacturers of America, GMA) in the Codex process, with many food companies working through these organizations.

2.6.2 ISO Standards

In addition to food standards and policies established by the Codex Alimentarius Commission, the International Organization for Standardization (ISO) has a series (9000 and beyond) of international standards on quality performance and record keeping (48–50). The intent of the quality series standards for a company is to establish quality systems, maintain product integrity, and satisfy customers. ISO focuses on documentation of procedures that ensure a systematic approach to quality management. Companies can elect to become registered only in the relevant parts of the ISO standards. Some manufacturers and retailers require food indus-

try suppliers to be ISO certified. Relevant to food analysis, ISO standards include sampling procedures and food standards.

2.6.3 Other Standards

Other international, regional, and country-specific organizations publish standards relevant to food composition and analysis. For example, the Saudi Arabian Standards Organization (SASO) publishes standards documents (e.g., labeling, testing methods) important in the Middle East (except Israel), and the European Commission sets standards for foods and food additives for countries in the European Economic Community (EEC). In the United States, the Food Chemicals Codex (FCC) Committee, which operates as part of the Food and Nutrition Board of the National Academy of Sciences, sets standards for the identification and purity of food additives and chemicals (51). For example, a company may specify in the purchase of a specific food ingredient that it be "FCC grade." Countries other than the United States adopt FCC standards (e.g., Australia, Canada). At an international level, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) sets standards for purity of food additives (52). The Codex Alimentarius Commission is encouraged to utilize the standards established by JECFA. Standards established by FCC and JECFA are used by many countries as they develop their own standards.

2.7 SUMMARY

Various kinds of standards set for certain food products by federal agencies make it possible to get essentially the same food product whenever and wherever purchased in the United States. The standards of identity set by the FDA and USDA define what certain food products must consist of. The USDA and National Marine Fisheries Service of the Department of Commerce have specified grade standards to define attributes for certain foods. Grading programs are voluntary, while inspection programs may be either voluntary or mandatory, depending on the specific food product.

While the FDA has broadest regulatory authority over most foods, responsibility is shared with other regulatory agencies for certain foods. The USDA has significant responsibilities for meat and poultry; the National Oceanic and Atmospheric Administration and the National Marine Fisheries Service for seafood; and the Bureau of Alcohol, Tobacco, and Firearms for alcoholic beverages. The FDA, the USDA, state agencies, and the dairy industry work together to ensure the safety, quality, and economic integrity of milk and milk products. The FDA, the EPA, and state agencies

work together in the National Shellfish-Sanitation Program to ensure the safety and wholesomeness of shellfish. The EPA shares responsibility with the FDA for control of pesticide residues in foods and has responsibility for drinking water safety and the composition of effluent from food processing plants. The Customs Service receives assistance from the FDA and USDA in its role to ensure the safety and economic integrity of imported foods. The Federal Trade Commission works with the FDA to prevent deceptive advertising of food products, as affected by food composition and labels. The National Conference on Weights and Measures, under the National Institute of Standards and Technology within the Department of Commerce, has developed model packaging and labeling regulations related to weights and measures of food packages.

The chemical composition of foods is often an important factor in determining the quality, grade, and price of a food. Government agencies that purchase foods for special programs often rely on detailed specifications that include information on food composition.

International organizations have developed food standards and safety practices to protect consumers, ensure fair business practices, and facilitate international trade. The Codex Alimentarius Commission is the major international standard-setting group for food safety and quality. The International Organization for Standardization has a series of standards that focus on documentation of procedures, with some relevant to food analysis. Certain regional and country-specific organizations also publish standards related to food composition and analysis.

2.8 STUDY QUESTIONS

1. Define the abbreviations FDA, USDA, and EPA, and give two examples for each of what they do or regulate relevant to food analysis.
2. Differentiate "standards of identity," "standards of quality," and "grade standards" with regard to what they are and which federal agency establishes and regulates them.
3. Government regulations regarding the composition of foods often state the official or standard method by which the food is to be analyzed. Give the full name of three organizations that publish commonly referenced sources of such methods.
4. For each type of product listed below, identify the governmental agency (or agencies) that has regulatory or other responsibility for quality assurance. Specify the nature of that responsibility.
   a. frozen fish sticks
   b. contaminants in drinking water
   c. dessert wine
   d. Grade A milk
   e. frozen oysters
   f. imported chocolate products

g. residual pesticide on wheat grain
h. corned beef
5. Food products purchased by federal agencies often have specifications that include requirements for chemical composition. Give the names of four such specifications.
6. You are developing a food product that will be marked in another country. What factors will you consider as you decide what ingredients to use and what information to include on the food label? What resources should you use as you make these decisions?

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2.10 RELEVANT INTERNET ADDRESSES

American Public Health Association
  http://www.apha.org/
American Society of Testing Materials
  http://www.astm.org
AOAC International
  http://www.aoac.org
Bureau of Alcohol, Tobacco and Firearms
  http://www.atf.treas.gov/
Centers for Disease Control and Prevention
  http://www.cdc.gov/
Code of Federal Regulations
  http://www.access.gpo.gov/nara/cfr/
    cfr-table-search.html
Codex Alimentarius Commission
  http://www.fao.org/waicent/faoinfo/
    economic/esn/codex/codex.htm
Department of Commerce
  http://www.doc.gov/
  National Institute of Standards and Technology
    http://www.nist.gov/
    National Conference on Weights and Measures
      http://ts.nist.gov/ts/htdocs/230/235/owm
      home.htm#name-3
  National Oceanic and Atmospheric Administration
    http://www.noaa.gov/

National Marine Fisheries Service
  http://kingfish.ssp.nmfs.gov/
Environmental Protection Agency
  http://www.epa.gov/
Federal Trade Commission
  http://www.ftc.gov/
Food Chemicals Codex
  http://www2.nas.edu/codex
Food and Drug Administration
  http://www.fda.gov
  Center for Food Safety & Applied Nutrition
    http://vm.cfsan.fda.gov/list.html
    Food Labeling and Nutrition
      http://vm.cfsan.fda.gov/label.html
    Hazard Analysis Critical Control Point
      http://vm.cfsan.fda.gov/~lrd/haccpsub.
      html
    Milk Safety References
      http://vm.cfsan.fda.gov/~ear/prime.html
    Pesticides, Metals & Industrial Chemicals
      http://vm.cfsan.fda.gov/~lrd/pestadd.
      html
    Seafood Information and Resources
      http://vm.cfsan.fda.gov/seafood1.html
International Organization for Standardization
  http://www.iso.ch/
National Shellfish Sanitation Program
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U.S. Customs Service
  http://www.customs.ustreas.gov/
U.S. Department of Agriculture
  http://www.usda.gov
  Agricultural Marketing Service
    http://www.ams.usda.gov/
    Quality Standards
      http://www.ams.usda.gov/standards/
    Laboratory Testing Program
      http://www.ams.usda.gov/labserv.htm
  Food Safety and Inspection Service
    http://www.usda.gov/fsis
    HACCP/Pathogen Reduction
      http://www.usda.gov/agency/fsis/
      imphaccp.htm
  Grain Inspection, Packers and Stockyards Administration
    http://www.usda.gov/gipsa

ACKNOWLEDGMENTS

Nutrition Labeling

S. Suzanne Nielsen

3.1 INTRODUCTION

Nutrition labeling regulations differ in countries around the world. The focus of this chapter is on nutrition labeling regulations in the United States, as specified by the Food and Drug Administration (FDA) and the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture (USDA). A major reason for analyzing the chemical components of foods in the United States is nutrition labeling regulations. The FDA was authorized under the 1906 Federal Food and Drug Act and the 1938 Federal Food, Drug, and Cosmetic (FD&C) Act to require certain types of food labeling (1, 2). This labeling information includes the amount of food in a package, its common or usual name, and its ingredients. The 1990 Nutrition Labeling and Education Act (NLEA) (2, 3) modified the 1938 FD & C Act to regulate nutrition labeling.

The FDA and FSIS of the USDA (referred to throughout chapter simply as FSIS) have coordinated their regulations for nutrition labeling. The regulations of both agencies strive to follow the intent of the NLEA, although only the FDA is bound by the legislation. The differences that exist in the regulations are due principally to the inherent differences in the food products regulated by the FDA and USDA (USDA regulates meat, poultry, and meat and poultry products, only). The two agencies maintain close harmony regarding interpretation of the regulations and changes made in regulations.

Complete details of the current nutrition labeling regulations are available in the Federal Register and the Code of Federal Regulations (CFR) (4–7). The 1973 regulations on nutrition labeling and the 1990 NLEA are described briefly below, followed by select aspects of current FDA and FSIS nutrition labeling regulations. In developing a nutrition label for a food product, refer to details of the regulations in the CFR and other references cited. A reference manual that explains nutritional labeling regulations (with continual updating) can be purchased from the National Food Processors Association (a nonprofit organization) (8) and several commercial publishers (9). The FDA has available in print and on the Internet *A Food Labeling Guide* (10), which refers to the document *Food Labeling—Questions and Answers*, available from the Industry Activities Staff (11). This and other relevant Internet addresses are given at the end of this chapter.

3.1.1 1973 Regulations on Nutrition Labeling

The FDA promulgated regulations in 1973 that permitted, and in some cases required, foods to be labeled with regard to their nutritional value (1, 2). Nutrition labeling was required only if a food contained an added nutrient or if a nutrition claim was made for the

food on the label or in advertising. The nutrition label included the following: serving size; number of servings per container; calories per serving; grams of protein, carbohydrate, and fat per serving; and percentage of U.S. Recommended Dietary Allowance (USRDA) per serving of protein, Vitamins A and C, thiamine, riboflavin, niacin, calcium, and iron. In 1984, the FDA adopted regulations to include sodium content on the nutritional label (effective 1985).

3.1.2 Nutrition Labeling and Education Act (NLEA) of 1990

Since the nutrition label was established in 1973, dietary ecommendations for better health have focused more on the role of calories and macronutrients (fat, carbohydrates, etc.) in chronic diseases and less on the role of micronutrients (minerals and vitamins) in deficiency diseases. Therefore, in the early 1990s the FDA revised the content of the nutritional label to make it more consistent with current dietary concerns (see Table 3-1 and Fig. 3-1, which are discussed more in section 3.2.1). The list of specific nutrients to be included on the nutrition label was only one aspect of the Nutrition Labeling and Education Act of 1990 (NLEA) (2, 3), which amended the FD&C Act with regard to five primary changes:

1. Mandatory nutrition labeling for almost all food products
2. Federal regulation of nutrient content claims and health claims
3. Authority for states to enforce certain provisions of FD&C Act
4. Federal preemption over state laws for misbranding provisions
5. Declaration of ingredients

3.2 FOOD LABELING REGULATIONS

For each aspect of nutrition labeling regulations described below, general or FDA labeling requirements are covered, followed by, if applicable, certain FSIS regulations that differ from the FDA requirements. While the focus here is on mandatory nutrition labeling, it should be noted that the FDA has guidelines for voluntary nutrition labeling of raw fruit, vegetables, and fish (21 CFR 101.45), and FSIS has guidelines for voluntary nutrition labeling of single-ingredient raw meat and poultry products (9 CFR 317.345, 317.445). These FDA and FSIS guidelines for voluntary nutrition labeling differ in issues such as source of nutrient databases used, compliance checks, and use of claims on product labels.

3.2.1 Mandatory Nutrition Labeling

3.2.1.1 Basic Format

The FDA regulations implementing the 1990 NLEA require nutrition labeling for most foods offered for sale and regulated by the FDA (21 CFR 101.9 to 101.108), and FSIS regulations require nutrition labeling of most meat or meat products (9 CFR 317.300 to 317.400) and poultry products (9 CFR 381.400 to 381.500). Certain nutrient information is required on the label, and other information is voluntary (Table 3-1). In addition, while FSIS allows voluntary declaration of stearic acid content on the label, FDA does not, but has been petitioned to do so.

The standard format for nutrition information on food labels [21 CFR 101.9 (d)] is given in Fig. 3-1 and consists of the following:

1. Serving size;
2. Quantitative amount per serving of each nutrient except vitamins and minerals;
3. Amount of each nutrient, except sugars and protein, as a percent of the Daily Value (i.e., the

new label reference values) for a 2000 calorie diet; and

4. Footnote with Daily Values for selected nutrients based on 2000 calorie and 2500 calorie diets.

The term Daily Value used on the basic label format refers to the two terms, Reference Daily Intake (RDI) and Daily Reference Value (DRV). The term RDI has replaced the term U.S. Recommended Dietary Allowance (USRDA). The RDI values for essential vitamins and minerals are given in Table 3-2 in the order in which they are to appear on nutrition labels, and the DRVs for food components are given in Table 3-3. A Daily Value for sugars has not been established. Nutrient content values and percent Daily Value calculations for the nutrition label are based on serving size. Serving size regulations of the FDA and FSIS differ in issues such as product categories, reference amounts, and serving size for units or pieces [21 CFR 101.12 (b), 101.9 (b); 9 CFR 317.312 (b), 381.412 (b), 317.309 (b), 381.409 (b)].

3-1 table Mandatory (Bold) and Voluntary Components for Food Label Under Nutrition Labeling and Education Act of 1990[1]

Total Calories
Calories from fat
Calories from saturated fat
Total fat
  Saturated fat
  Polyunsaturated fat
  Monounsaturated fat
Cholesterol
Sodium
Potassium
Total carbohydrate
  Dietary fiber
  Soluble fiber
  Insoluble fiber
  Sugars
  Sugar alcohols (e.g., sugar substitutes xylitol, mannitol, and sorbitol)
Other carbohydrate (the difference between total carbohydrate and the sum of dietary fiber, sugars, and sugar alcohols, if declared)
Protein
Vitamin A
Vitamin C
Calcium
Iron
Other essential vitamins and minerals

From (5).
[1]Nutrition panel will have the heading "Nutrition Facts." Only components listed are allowed on the nutrition panel, and they must be in the order listed. Components are to be expressed as amount and/or as percent of an established "Daily Value."

3-2 table Reference Daily Intakes (RDIs) for Vitamins and Minerals Essential in Human Nutrition[1,2]

Nutrient	RDI
Vitamin A	5000 IU
Vitamin C	60 mg
Calcium	1000 mg
Iron	18 mg
Vitamin D	400 IU
Vitamin E	30 IU
Vitamin K	80 µg
Thiamin	1.5 mg
Riboflavin	1.7 mg
Niacin	20 mg
Vitamin B$_6$	2 mg
Folate	400 µg
Vitamin B$_{12}$	6 µg
Biotin	300 µg
Pantothenic acid	10 mg
Phosphorus	1000 mg
Iodine	150 µg
Magnesium	400 mg
Zinc	15 mg
Selenium	70 µg
Copper	2 mg
Manganese	2 mg
Chromium	120 µg
Molybdenum	75 µg
Chloride	3400 mg

From 21 CFR 101.9 (c) (8) (iv) (1997).
[1]Values are for adults and children 4 or more years of age. RDI values have not so been established for infants, children under 4 years of age, and pregnant and lactating women.
[2]RDI values listed by the Food Safety and Inspection Service [9 CFR 317.309 (c) (8) (iv); 9 CFR 381.409 (c) (8) (iv)] are as above but do not include values for chloride, chromium, manganese, Vitamin K, molybdenum, and selenium.

3-3 table

Daily Reference Values (DRVs) of Food Components Based on the Reference Calorie Intake of 2000 Calories

Food Component	DRV
Fat	65 g
Saturated fatty acids	20 g
Cholesterol	300 mg
Total carbohydrate	300 g
Fiber	25 g
Sodium	2400 mg
Potassium	3500 mg
Protein	50 g

From 21 CFR 101.9 (c) (9) (1997). Same as in 9 CFR 317.309 (c) (9) and 9 CFR 381.409 (c) (9).

3.2.1.2 Simplified Format

A simplified format for nutrition information on FDA-regulated foods may be used if seven or more of the 13 required nutrients are present in only insignificant amounts (but does not include calories from fat) (e.g., soft drinks) [21 CFR 101.9 (f)]. For such foods, information on five core nutrients (calories, total fat, total carbohydrate, protein, and sodium) must be given. However, if other mandatory nutrients are present in more than insignificant amounts they must be listed. "Insignificant" is defined generally as the amount that allows a declaration of zero on the nutrition label. However, in the cases of protein, total carbohydrate, and dietary fiber, insignificant is the amount that allows a statement of "less than 1 gram." Footnotes to the simplified format label are optional, except if the complete footnote regarding percent DV is omitted, in which case the statement "Percent Daily Values are based on a 2000 calorie diet" must be included. The statement "Not a significant source of _____" is optional on the simplified format label of an FDA-regulated product, unless a nutrient claim is made on the label or optional nutrients (e.g., potassium) are voluntarily listed on the nutrition label.

For USDA-regulated foods, a simplified nutrition label format may be used when any nutrient other than a core nutrient (calories, total fat, sodium, carbohydrate, or protein) is present in an insignificant amount [9 CFR 317.309 (f), 381.409 (f)]. Missing nutrients must be listed in a footnote, "Not a significant source of _____." This option also exists for FDA-regulated foods but it is known as a "shortened" format [21 CFR 101.96 (c); see listing for each non-core nutrient].

3.2.1.3 Exemptions

Certain foods are exempt from FDA mandatory nutrition labeling requirements [21 CFR 101.9 (j)] (Table 3-4), unless a nutrient content claim or health claim is made. Special labeling provisions apply to certain other foods

Nutrition Facts

Serving Size 1 cup (228g)
Servings Per Container 2

Amount Per Serving

Calories 260   Calories from Fat 120

	% Daily Value*
Total Fat 13g	**20%**
Saturated Fat 5g	**25%**
Cholesterol 30mg	**10%**
Sodium 660mg	**28%**
Total Carbohydrate 31g	**10%**
Dietary Fiber 0g	**0%**
Sugars 5g	
Protein 5g	

Vitamin A 4%	•	Vitamin C 2%
Calcium 15%	•	Iron 4%

* Percent Daily Values are based on a 2,000 calorie diet. Your daily values may be higher or lower depending on your calorie needs:

	Calories:	2,000	2,500
Total Fat	Less than	65g	80g
Sat Fat	Less than	20g	25g
Cholesterol	Less than	300mg	300mg
Sodium	Less than	2,400mg	2,400mg
Total Carbohydrate		300g	375g
Dietary Fiber		25g	30g

Calories per gram:
Fat 9 • Carbohydrate 4 • Protein 4

3-1 figure

An example of the nutrition label, Nutrition Labeling and Education Act of 1990. (Courtesy of the Food and Drug Administration, Washington, DC.)

as specified in 21 CFR (e.g., foods in small packages; foods for children; game meats, shell eggs; foods sold from bulk containers; unit containers in multiunit packages; foods in gift packs). Infant formula must be labeled in accordance with 21 CFR 107, and raw fruits, vegetables, and fish according to 21 CFR 101.45. Dietary supplements must be labeled in accordance with 21 CFR 101.36 after March 1998.

Foods Exempt from Mandatory Nutrition Labeling Requirements by the FDA[1]

Food offered for sale by small business
Food sold in restaurants or other establishments in which food is served for immediate human consumption
Foods similar to restaurant foods that are ready to eat but are not for immediate consumption, are primarily prepared on site, and are not offered for sale outside that location
Foods that contain insignificant amounts of all nutrients subject to this rule, e.g., coffee and tea
Dietary supplements
Infant formula
Medical foods
Foods shipped or sold in bulk form and not for sale to consumers
Raw fruits, vegetables, and fish
Unit containers in a multiunit retail food package that bears a nutrition label

Summarized from 21 CFR 101.9 (j) (1997).
[1]See details in regulations.

Exemptions from mandatory nutrition labeling for USDA-regulated foods [9 CFR 317.400 (a), 381.500 (a)] differ somewhat from those for FDA-regulated foods regarding issues such as definitions of a small business, small package, and retail product.

3.2.1.4 Rounding Rules

Increments for the numerical expression of quantity per serving are specified for all mandatory nutrients (Table 3-5, as summarized by FDA) [21 CFR 101.9 (c); 9 CFR 317.309 (c), 381.409 (c)]. For example, calories are to be reported to the nearest 5 calories up to and including 50 calories, and to the nearest 10 calories above 50 calories. Calories can be reported as zero if there are less than 5 calories per serving.

3.2.1.5 Calorie Content

Caloric conversion information on the label for fat, carbohydrate, and protein is optional. The FDA regulations specify five methods by which caloric content may be calculated, one of which uses bomb calorimetry [21 CFR 101.9 (c) (1)]:

1. Specific Atwater factors for calories per gram of protein, total carbohydrate, and total fat;
2. The general factors of 4, 4, and 9 calories per gram of protein, total carbohydrate, and total fat, respectively;
3. The general factors of 4, 4, and 9 calories per gram of protein, total carbohydrate, less the amount of insoluble dietary fiber, and total fat, respectively;

4. Data for specific food factors for particular foods or ingredients approved by the FDA; or
5. Bomb calorimetry data subtracting 1.25 calories per gram protein to correct for incomplete digestibility.

FSIS allows only the calculation procedures 1–4 above, and not the use of bomb calorimetry for caloric content [9 CFR 317.309 (c) (1) (i), 381.409 (c) (1) (i)].

3.2.1.6 Protein Quality

For both FDA-regulated and USDA-regulated foods, reporting the amount of protein as a percent of its Daily Value is optional, except if a protein claim is made for the product, or if the product is represented or purported to be used by infants or children under four years of age, in which case the statement is required [21 CFR 101.9 (c) (7); 9CFR 317.309 (c) (7) (i), 381.409 (c) (7) (i)]. For infants foods, the corrected amount of protein per serving is calculated by multiplying the actual amount of protein (g) per serving by the relative protein quality value. This relative quality value is the Protein Efficiency Ratio (PER) value of the subject food product divided by the PER value for casein. For foods represented or purported for adults and children one year or older, the corrected amount of protein per serving is equal to the actual amount of protein (g) per serving multiplied by the Protein Digestibility—Corrected Amino Acid Score (PDCAAS). Both the PER and PDCAAS methods to assess protein quality are described in Chapter 17. The FDA and FSIS allow use of the general factor 6.25 and food specific factors for this calculation (described in Chapter 15).

3.2.2 Compliance

Compliance procedures of the FDA and FSIS for nutrition labeling differ somewhat in sample collection, specified methods of analysis, and levels required for compliance [21 CFR 101.9 (g); 9 CFR 317.309 (h), 381.409 (h)].

3.2.2.1 Sample Collection

Random sampling techniques are used by the FDA to collect samples to be analyzed for compliance with nutrition labeling regulations. A "lot" is the basis for sample collection by the FDA, defined as "a collection of primary containers or units of the same size, type, and style produced under conditions as nearly uniform as possible, and designated by a common container code or marking, or in the absence of any common container code or marking, a day's production." The sample used by the FDA for nutrient analysis con-

3-5
table

Rounding Rules for Declaring Nutrients on Nutrition Label[1]

Nutrient/Serving	Increment Rounding	Insignificant Amount
Calories	<5 cal—express as zero ≤50 cal—express to nearest 5 cal increment >50 cal—express to nearest 10 cal increment	<5 cal
Calories from fat	<5 cal—express as zero ≤50 cal—express to nearest 5 cal increment >50 cal—express to nearest 10 cal increment	<5 cal
Calories from saturated fat	<5 cal—express as zero ≤50 cal—express to nearest 5 cal increment >50 cal—express to nearest 10 cal increment	<5 cal
Total fat, Polysaturated fat, Monounsaturated fat	<0.5 g—express as zero <5 g—express to nearest 0.5 g increment ≥5 g—express to nearest 1 g increment	<0.5 g
Saturated fat	<0.5 g—express as zero <5 g—express to nearest 0.5 g increment ≥5 g—express to nearest 1 g increment	<0.5 g
Cholesterol	<2 mg—express as zero 2-5 mg—express as "less than 5 mg" >5 mg—express to nearest 5 mg increment	<2 mg
Sodium, Potassium	<5 mg—express as zero 5-140 mg—express to nearest 5 mg increment >140 mg—express to nearest 10 mg increment	<5 mg
Total carbohydrate, Sugars, Sugar alcohols, Other carbohydrates	<0.5 g—express as zero <1 g—express as "Contains less than 1 g" OR "less than 1 g" ≥1 g—express to nearest 1 g increment	<1 g
Dietary fiber, Soluble fiber, Insoluble fiber	<0.5 g—express as zero <1 g—express as "Contains less than 1 g" OR "less than 1 g" ≥1 g—express to nearest 1 g increment	<1 g
Protein	<0.5 g—express as zero <1 g—express as "Contains less than 1 g" OR "less than 1 g" ≥1 g—express to nearest 1 g increment	<1 g
Vitamins and Minerals	<2% of RDI—may be expressed as: (1) 2% if actual amount is 1.0% or more (2) zero (3) an asterisk that refers to statement "Contains less than 2% of the Daily Value of this (these) nutrient (nutrients) (4) for Vitamins A and C, calcium, iron: statement "Not a significant source of ___ (listing the vitamins or minerals omitted)" ≤10% of RDI—express to nearest 2% increment >10% to ≤50% of Vitamin A—express to nearest 5% increment >50% of RDI—express to nearest 10% increment	<2% RDI
Beta-carotene	≤10% of Vitamin A—express to nearest 2% increment >10% to ≤50% of Vitamin A—express to nearest 5% increment >50% of Vitamin A—express to nearest 10% increment	

[1]Summarized from technical amendments of mandatory nutritional labeling final rule, August 1993. FDA. Dec. 4, 1994. Office of Food Labeling, Center for Food Safety and Applied Nutrition, Food and Drug Administration, Washington, DC.

[2]To express to the nearest 1 g increment, amounts exactly halfway between two whole numbers or higher (e.g., 2.50 to 2.99 g) round up (e.g., 3 g), and amounts less than halfway between two whole numbers (e.g., 2.01 to 2.49 g) round down (e.g., 2 g).

[3]NOTES FOR ROUNDING % Daily Value (DV) for Total Fat, Saturated Fat, Cholesterol, Sodium, Total Carbohydrate, Fiber, and Protein:

(a) To calculate %DV, divide either the actual (unrounded) quantitative amount or the declared (rounded) amount by the appropriate RDI or DRV. Use whichever amount will provide the greatest consistency on the food label and prevent unnecessary consumer confusion (21 CFR 101.9 (d)(7)(ii).

(b) When %DV values fall between two whole numbers, rounding shall be as follows:

—for values exactly halfway between two whole numbers or higher (e.g., 2.50 to 2.99) the values shall round up (e.g., 3%).

—for values less than halfway between two whole numbers (e.g., 2.01 to 2.49) the values shall round down (e.g., 2%).

sists of a "composite of 12 subsamples (consumer units), taken 1 from each of 12 different randomly chosen shipping cases, to be representative of a lot" [21 CFR 101.0 (g)].

The FSIS defines a "lot" similar to that of the FDA. However, the sample used by FSIS for compliance analysis is a composite of a minimum of six consumer units: (1) each from a production lot, or (2) each randomly chosen to be representative of a production lot [9 CRF 317.309 (h), 381.409 (h)].

3.2.2.2 Methods of Analysis

The FDA states that unless a particular method of analysis is specified in 21 CFR 101.9(c), appropriate methods of AOAC International published in the *Official Methods of Analysis* (12) are to be used. Other reliable and appropriate methods can be used if no AOAC method is available or appropriate. If scientific knowledge or reliable databases have established that a nutrient is not present in a specific product (e.g., dietary fiber in seafood, cholesterol in vegetables), the FDA does not require analyses for the nutrients. FSIS specifies for nutritional analysis the methods of the USDA *Chemistry Laboratory Guidebook* (13). If no USDA method is available and appropriate for the nutrient, methods in the *Official Methods of Analysis* of AOAC International (12) are to be used. If no USDA, AOAC International, or specified method is available and appropriate, FSIS specifies the use of other reliable and appropriate analytical procedures as determined by the Agency.

3.2.2.3 Levels for Compliance

The FDA and FSIS both monitor accuracy of nutrient content information for compliance based on two classes of nutrients and an unnamed third group, as described in Table 3-6. For example, a product fortified with iron would be considered misbranded if it contained less than 100% of the label declaration. A product that naturally contains dietary fiber would be considered misbranded if it contained less than 80% of the label declaration. A product would be considered misbranded if it had a caloric content greater than 20% in excess of the label declaration. Reasonable excesses over labeled amounts (of a vitamin, mineral, protein, total carbohydrate, polyunsaturated or monounsaturated fat, or potassium) or deficiencies below label amounts (of calories, sugars, total fat, saturated fat, cholesterol, or sodium) are acceptable within current Good Manufacturing Practices (cGMP).

For FDA-regulated foods, compliance with the regulations described above can be obtained by use of FDA-approved databases (14) [21 CFR 109.9 (g) (8)] that have been computed using FDA guidelines, and foods have been handled under cGMP conditions to

Basis for Compliance of Nutrition Labeling Regulation by Food and Drug Administration and Food Safety and Inspection Service of the United States Department of Agriculture[1]

Class of Nutrients	Purposes of Compliance	Nutrients Regulated	% Required[3]
I	Added nutrients in fortified or fabricated foods	Vitamins, minerals, protein, dietary fiber, potassium	≥100%
II	Naturally occurring (indigenous) nutrients	Vitamins, minerals, protein, total carbohydrate, dietary fiber, other carbohydrates, polyunsaturated or monounsaturated fat, potassium	≥80%
•2		Calories, sugar, total fat, saturated fat, cholesterol, sodium	≤120%

[1]Summarized from 21 CFR 101.9 (g), 9 CFR 317.309 (h), and 9 CFR 381.409(h) (1997).
[2]•Unnamed class.
[3]Amount of nutrient required in food sample as a percentage of the label declaration, or else product is considered misbranded.

prevent nutritional losses. For USDA-regulated foods, compliance enforcement described previously is not applicable to single-ingredient, raw meat products (including those frozen previously), when nutrition labeling is based on database values in USDA's National Nutrient Data Bank or in USDA Handbook No. 8 (most recent version on the Internet) (15). The USDA does not preapprove databases, but provides a manual for guidance in using them (16).

3.2.3 Nutrient Content Claims

The FDA and FSIS have defined nutrient descriptors, which are nutrient content claims that characterize the level of a nutrient but do not include nutrient labeling information or disease prevention claims. Nutrient descriptors are based on definitions that require certain types of nutrient analyses to determine the levels of the nutrients related to the descriptor. The terms include "free," "low," "lean," "light," "lite," "reduced," "less," "good source," "more," and "high" (21 CFR 101.13, 101.54-101.67; 9 CFR 317.313, 317.354-317.363, 381.413, 381.454-381.463) (Tables 3-7 and 3-8 are FDA regula-

Nutrients	Free	Low	Reduced/Less	Comments[1]
	• Synonyms for "Free": "Zero," "No," "Without," "Trivial Source of," "Negligible Source of,". "Dietarily Insignificant Source of" • Definitions for "Free" for meals and main dishes are the stated values per labeled serving.	• Synonyms for "Low": "Little," ("Few" for calories), "Contains a Small Amount of," "Low Source of"	• Synonyms for "Reduced"/"Less": "Lower" ("Fewer" for calories) • "Modified" may be used in statement of identity. • Definitions for meals and main dishes are same as for individual foods on a per 100 g basis.	• For "Free," "Very Low," or "Low": must indicate if food meets a definition without benefit of special processing, alteration, formulation, or reformulation; e.g., "broccoli, a fat free food," or "celery, a low calorie food."
Calories § 101.6C(b)	• Less than 5 calories per reference amount and per labeled serving • Not defined for meals or main dishes	• 40 calories or less per reference amount (and per 50 g if reference amount is small) • Meals and main dishes: 120 cal or less per 100 g	• At least 25% fewer calories per reference amount than an appropriate reference food • Reference food may not be "Low Calorie" • Uses term "Fewer" rather than "Less"	• "Light" or "Lite": If 50% or more of the calories are from fat, fat must be reduced by at least 50% per reference amount. If less than 50% of calories are from fat, fat must be reduced at least 50% or calories reduced at least 1/3 per reference amount. • "Light" or "Lite" meal or main dish product meets definition for "Low Calorie" or "Low Fat" meal and is labeled to indicate which definition is met. • For dietary supplements: Calorie claims can only be made when the reference product is greater than 40 calories per serving.
Total Fat § 101.62(b)	• Less than 0.5 g per reference amount and per labeled serving (or for meals and main dishes, less than 0.5 g per labeled serving) • No ingredient that is fat or understood to contain fat except as noted below*	• 3 g or less per reference amount (and per 50 g if reference amount is small) • Meals and main dishes: 3 g or less per 100 g and not more than 30% of calories from fat	• At least 25% less fat per reference amount than an appropriate reference food • Reference food may not be "Low Fat"	• "___% Fat Free": OK if food meets the requirements for "Low Fat" • "Light": See above • For dietary supplements: Fat claims cannot be made for products that are 40 calories or less per serving.
Saturated Fat § 101.62(c)	• Less than 0.5 g saturated fat and less than 0.5 *trans* fatty acids per reference amount and per labeled serving (or for meals and main dishes, less than 0.5 g saturated fat and less than 0.5 g *trans* fatty acid per labeled serving) • No ingredient that is understood to contain saturated fat except as noted below*	• 1 g or less per reference amount and 15% or fewer calories from saturated fat • Meals and main dishes: 1 g or less per 100 g and less than 10% of calories from saturated fat	• At least 25% less saturated fat per reference amount than an appropriate reference food • Reference food may not be "Low Saturated Fat"	• Next to all saturated fat claims, must declare the amount of cholesterol if 2 mg or more per reference amount; and the amount of total fat if more than 3 g per reference amount (or 0.5 g or more of total fat for "Saturated Fat Free").[3] • For dietary supplements: Saturated fat claims cannot be made for products that are 40 calories or less per serving.

(continued)

Food and Drug Administration: Definitions of Nutrient Content Claims (continued)

Nutrients	Free	Low	Reduced/Less	Comments[1]
Cholesterol § 101.62(d)	• Less than 2 mg per reference amount and per labeled serving (or for meals and main dishes, less than 2 mg per labeled serving) • No ingredient that contains cholesterol except as noted below* • If less than 2 mg per reference amount by special processing and total fat exceeds 13 g per reference amount and labeled serving, the amount of cholesterol must be "Substantially Less" (25%) than in a reference food with significant market share (5% of market).	• 20 mg or less per reference amount (and per 50 g of food if reference amount is small) • If qualifies by special processing and total fat exceeds 13 g per reference amount and labeled serving, the amount of cholesterol must be "Substantially Less" (25%) than in a reference food with significant market share (5% of market). • Meals and main dishes: 20 mg or less per 100 g	• At least 25% less cholesterol per reference amount than an appropriate reference food • Reference food may not be "Low Cholesterol"	• CHOLESTEROL CLAIMS ONLY ALLOWED WHEN FOOD CONTAINS 2 g OR LESS SATURATED FAT PER REFERENCE AMOUNT, OR FOR MEALS AND MAIN DISH PRODUCTS, PER LABELED SERVING SIZE FOR "FREE" CLAIMS AND PER 100 g FOR "LOW" AND "REDUCED/LESS" CLAIMS • Must declare the amount of total fat next to cholesterol claim when fat exceeds 13 g per reference amount of labeled serving (or per 50 g of food if reference amount is small), or when the fat exceeds 19.5 g per labeled serving for main dishes or 26 g for meal products. • For dietary supplements: cholesterol claims cannot be made for products that are 40 calories or less per serving.
Sodium § 101.61	• Less than 5 mg per reference amount and per labeled serving (or for meals and main dishes, less than 5 mg per labeled serving) No ingredient that is sodium chloride or generally understood to contain sodium except as noted below*	• 140 mg or less per reference amount (and per 50 g if reference amount is small) • Meals and main dishes: 140 mg or less per 100 g	• At least 25% less sodium per reference amount than an appropriate reference food • Reference food may not be "Low Sodium"	• "Light" (for sodium reduced products): If food is "Low Calorie" and "Low Fat" and sodium is reduced by at least 50% • "Light in Sodium": If sodium is reduced by at least 50% per reference amount. Entire term "Light in Sodium" must be used in the same type size, color, and prominence. "Light in Sodium" for meals = "Low in Sodium" • "Very" Low Sodium: 35 mg or less per reference amount (and per 50 g if reference amount is small). For meals and main dishes: 35 mg or less per 100 g • "Salt Free" must meet criterion for "Sodium Free" • "No Salt Added" and "Unsalted" must meet conditions of use and must declare "This is Not A Sodium Free Food" on information panel if food is not "Sodium Free"

3-7
table   Food and Drug Administration: Definitions of Nutrient Content Claims (continued)

Nutrients	Free	Low	Reduced/Less	Comments[1]
Sugars § 101.60(c)	• "Sugar Free": Less than 0.5 g sugars per reference amount and per labeled serving (or for meals and main dishes, less than 0.5 g per labeled serving) • No ingredient that is a sugar or generally understood to contain sugars except as noted below* • Disclose calorie profile (e.g., "Low Calorie").	• Not defined. No basis for a recommended intake	• At lease 25% less sugars per reference amount than an appropriate reference food • May not use this claim on dietary supplements of vitamins and minerals.	• "Lightly Salted": 50 % less sodium than normally added to reference food and if not "Low Sodium" so labeled on information • "No Added Sugars" and "Without Added Sugars" are allowed if no sugar or sugar containing ingredient is added during processing. State if food is not "Low" or "Reduced Calorie." • The terms "Unsweetened" and "No Added Sweeteners" remain as factual statements. • Claims about reducing dental caries are implied health claims. • Does not include sugar alcohols

From (10), p. 44, 45, updated.
Notes: *Except if the ingredient listed in the ingredient statement has an asterisk that refers to the footnote (e.g., "adds a trivial amount of fat").
•"Reference Amount" = reference amount customarily consumed.
•"Small Reference Amount" = reference amount of 30 g or less or 2 tablespoons or less (for dehydrated foods that are typically consumed when rehydrated with water or a diluent containing an insignificant amount, as defined in Sss 101.9(f)(1) of all nutrients per reference amount, the per 50 g criterion refers to the prepared form of the food).
•Statement "See ____ panel for nutrition information" must accompany all content claims. When levels exceed: 13 g Fat, 4 g Saturated Fat, 60 mg Cholesterol, or 480 mg Sodium per reference amount, per labeled serving or, for foods with small reference amounts, per 50 g, disclosure statement is required as part of claim (e.g., "See side panel for information on fat and other nutrients").

tions). The term "healthy" or its derivatives may be used on the label or in labeling of foods under conditions defined by the FDA and FSIS (Table 3-9 is FDA summary). The FDA requirements on nutrient content claims do not apply to infant formulas and medical foods.

Only nutrient descriptors defined by the FDA or FSIS may be used. The terms "less" (or "fewer"), "more," "reduced," "added" (or "fortified" and "enriched"), and "light/lite" are relative terms and require label information about the food product that is the basis of the comparison. The percentage difference between the original food and the food product being labeled must be listed on the label for comparison.

FSIS regulations on nutrient content claims differ from those of the FDA in that the terms "enriched" and "fortified" are not defined in the FSIS regulations (9 CFR 317.362, 381.462). The terms "lean" and "extra lean" are defined and approved for use on all USDA-regulated products, but only for the FDA-regulated products of seafood, game meat, and meal products. The term "____% Lean" is approved for USDA-regulated but not FDA-regulated products. The term

"____% Fat Free" is approved for use by both organizations as long as the food meets the definition for "low fat." Conditions for use of the term "light" or "lite" as part of a brand name to describe sodium content differ somewhat between FDA and USDA regulations [21 CFR 101.56 (b) (4); 9 CFR 356 (d) (3); 9 CFR 381.456 (d) (3)]. FDA and FSIS regulations also differ in the definition of "meal product" as it relates to nutrient content claims [21 CFR 101.13 (1) and (m); 9 CFR 317.313 (1), 318.413 (1)].

The referral statement "See ____ for nutrition information" (blank is filled in with identity of panel on which nutrition labeling is located) is required by the FDA whenever a nutrient content claim is made [21 CFR 101.13 (g) and (h)]. In addition, the FDA regulations specify disclosure levels that are required on foods when the product contains fat, saturated fat, cholesterol, or sodium at levels greater than 20%, 30%, and 40% of the DRV for individual foods, main dish items, and meals, respectively [21 CFR 101.13 (h)]. When one of these nutrients exceeds the disclosure level in a food bearing a nutrient content claim, the referral statement must be modified to say "See [appropriate panel] for

3-8 table
Food and Drug Administration: Nutrient Content Claims

Accompanying Information for Relative (or Comparative) Claims

- For all relative claims, percent (or fraction) of change and identity of reference food must be declared in immediate proximity to the most prominent claim. Quantitative comparison of the amount of the nutrient in the product per labeled serving with that in the reference food must be declared on the information panel.

- For "Light" claims: % reduction for both fat and calories must be stated out % reduction need not be specified if product is low in the nutrient. Quantitative comparisons must be stated for both fat and calories.

Reference Foods

"Light"/"Lite"	(1) A food representative of the type of food bearing the claim, e.g., average value of top three brands for representative value from valid data base; (2) Similar food (e.g., potato chips for potato chips); and (3) Not low calorie and low fat (except "light" or sodium reduced foods which *must* be low calorie and low fat).
"Reduced" and "Added" (or "Fortified" and "Enriched")	(1) An established regular product or average representative product and (2) Similar food
"More" and "Less" (or "Fewer")	(1) An established regular product or average representative product and (2) A dissimilar food in the same product category that may be generally substituted for labeled food (e.g., potato chips for pretzels) or a similar food.

Other Nutrient Content Claims

"Lean"	On seafood or game meat that contains <10 g total fat, 4.5 g or less saturated fat, and <95 mg cholesterol per reference amount and per 100 g (for meals and main dishes, meets criteria per 100 g and per label serving)
"Extra Lean"	On seafood or game meat that contains <5 g total fat, <2 g saturated fat, and <95 mg cholesterol per reference amount and per 100 g (for meals and main dishes, meets criteria per 100 g and per label serving)
"Good Source of," "Contains," or "Provides"	Contains 10% or more of Daily Value (DV) to describe protein, vitamins, minerals, dietary fiber, or potassium per reference amount. May be used on meals or main dishes to indicate that product contains a food that meets definition but may not be used to describe meal or main dish itself. May not be used for total carbohydrates.
"High," "Rich in," or "Excellent Source of"[1]	Contains 20% or more of the Daily Value (DV) to describe protein, vitamins, minerals, dietary fiber, or potassium per reference amount. May be used on meals or main dishes to indicate that product contains a food that meets definition but may not be used to describe meal or main dish itself. May not be used for total carbohydrates.
"More," "Added," "Extra," "Plus"[1]	10% or more of the DV per reference amount May only be used for vitamins, minerals, protein, dietary fiber, and potassium.
"Modified"	May be used in statement of identity that bears a relative claim, e.g., "Modified Fat Cheese Cake, Contains 35% Less Fat Than Other Regular Cheese Cake."
Any Fiber Claim	If food is not low in total fat, must state total fat in conjunction with claim such as "More Fiber."

[1]Dietary supplements cannot use these claims to describe any nutrient or ingredient (e.g., fiber, protein, psyllium, bran) other than vitamins or minerals.

Implied Claims

- Claims about a food or ingredient that suggest that the nutrient or ingredient is absent or present in a certain amount or claims about a food that suggest a food may be useful in maintaining healthy dietary practices and that are made with an explicit claim (e.g., "healthy, contains 3 grams of fat") are implied claims and are prohibited unless provided for in a regulation by FDA. In addition, the Agency has devised a petition system whereby specific additional claims may be considered.

- Claims that a food contains or is made with an ingredient that is known to contain a particular nutrient may be made if the product is "Low" in or a "Good Source" of the nutrient associated with the claim (e.g., "good source of oat bran").

3-8 table

Food and Drug Administration: Nutrient Content Claims (continued)

Implied Claims (continued)

- Equivalence claims: "Contains as much [nutrient] as a [food]" may be made if both reference food and labeled food are a "Good Source" of the nutrient on a per serving basis (e.g., "Contains as much vitamin C as an 8 ounce glass of orange juice").

- The following label statements are generally not considered implied claims unless they are made in a nutrition context: (1) avoidance claims for religious, food intolerance, or other non-nutrition related reasons [e.g., "100% milk free"]; (2) statements about non-nutritive substances [e.g., "no artificial color"]; (3) added value statements [e.g., "made with real butter"]; (4) statements of identity [e.g., "corn oil" or "corn oil margarine"]; and (5) special dietary statements made in compliance with a specific Part 105 provision.

Claims on Food for Infants and Children Less Than 2 Years of Age

Nutrient content claims are not permitted on foods intended specifically for infants and children less than 2 years of age except:
1. Claims describing the percentage of vitamins and minerals in a food in relation to a Daily Value
2. Claims on infant formulas provided for in Part 107
3. The terms "Unsweetened" and "Unsalted" as taste claims
4. "Sugar Free" and "No Added Sugar" claims on dietary supplements only

Terms Covered That Are Not Nutrient Content Claims

"Fresh"	A raw food that has not been frozen, heat processed, or otherwise preserved
"Fresh Frozen"	Food that was quickly frozen while still fresh

From (10), p. 46, 47, updated.

information about [nutrient requiring disclosure] and other nutrients [21 CFR 101.13 (h) (1)]. For USDA-regulated foods, no disclosure levels are defined or referral statement required except in the case of health claims (proposed rule) [9 CFR 317.371 (a)—(h)].

3.2.4 Health Claims

The FDA has defined and will allow as part of the 1990 NLEA claims for certain relationships (10 claims, as of 1997) between a nutrient or a food and the risk of a disease or health-related condition (21 CFR 101.14):

1. Calcium and osteoporosis (21 CFR 101.72)
2. Sodium and hypertension (21 CFR 101.74)
3. Dietary fat and cancer (21 CFR 101.73)
4. Dietary saturated fat and cholesterol and risk of coronary heart disease (21 CFR 101.75)
5. Fiber-containing grain products, fruits, and vegetables and cancer (21 CFR 101.76)
6. Fruits, vegetables, and grain products that contain fiber, particularly soluble fiber, and risk of coronary heart disease (21 CFR 101.77)
7. Fruits and vegetables and cancer (21 CFR 101.78)
8. Folate and neural tube defects (21 CFR 101.79)
9. Soluble fiber from whole oats and coronary heart disease [21 CFR 101.81]
10. Sugar alcohols and dental caries [21 CFR 101.80]

Such claims can be made through third-party references (e.g., National Cancer Institute), statements, symbols (e.g., heart), and vignettes or descriptions. The claim must meet the requirements for authorized health claims, and it must state that other factors play a role in that disease. The FDA is considering additional claims.

The FSIS has proposed regulations on health claims (59 FR 27144) and is considering further supplements to the proposed health claims that are very similar to the final rule regulations of the FDA. However, the proposed FSIS regulations have a higher disqualifying level for cholesterol in individual foods and meals, and regulations on use of the term "extra lean" with health claims differ from those of the FDA. Unlike the FDA, FSIS allows no sugar alcohol claim because it is not applicable to USDA-regulated foods.

3.2.5 National Uniformity and Preemption Authorized by NLEA

To provide for national uniformity, the 1990 NLEA authorizes federal preemption of certain state and local labeling requirements that are not identical to federal requirements. This pertains to requirements for food standards, nutrition labeling, claims of nutrient content, health claims, and ingredient declaration. States may petition the FDA for exemption of state requirements from federal preemption.

3-9 table   Conditions for Use of the Term "Healthy" in Labeling of Foods [1]

Individual Food	Seafood or Game Meat [2]	Meal or Main Dish
Total Fat		
Low Fat	<5 g fat per RA[3] and per 100 g	Low Fat
Saturated Fat		
Low Saturated Fat	<2 g fat per RA and per 100 g	Low Saturated Fat
Sodium (Before Jan. 2000)		
≤480 mg per RA, per LS[3] and per 50 g if RA[3] small	≤480 mg per RA and per LS and per 50 g if RA small[3]	≤600 mg per LS
Sodium (After Jan. 2000)		
≤360 mg per RA, per LS and per 50 g if RA small	≤360 mg per RA and per LS and per 50 g if RA small	≤480 mg per LS
Cholesterol		
≤Disclosure level	<95 mg per RA and per 100 g	≤90 mg per LS
Beneficial Nutrients		
Except raw fruits or vegetables, at least 10% of DV[3] per RA of Vitamin A, Vitamin C, calcium, iron, protein, or fiber	N/A	10% DV per LS of 2 nutrients for main dish, 3 nutrients for meal
Fortification		
Per 21 CFR 104.20	Per 21 CFR 104.20	Per 21 CFR 104.20
Other Claims		
Food complies with established definition and declaration requirements for any specific nutrient content claim.		

[1] From (10), p. 58, updated.
[2] Raw, single ingredient seafood or game meat once processed becomes an individual food, meal, or main dish.
[3] RA = Reference Amount, LS = Labeled Serving, DV = Daily Value. RA small = 30 g or less or 2 tablespoons or less.

3.2.6 Other Provisions of NLEA

The 1990 NLEA amends the FD&C Act to allow a state to bring, in its own name in state court, an action to enforce the food labeling provisions of the FD&C Act that are the subject of national uniformity. Criteria are defined for a state to exercise this enforcement power. The rule-making procedure for standards of identity was modified by the 1990 Act. The FD&C Act is also amended to impose several new requirements concerning ingredient labeling intended to make this aspect of labeling more useful to consumers.

3.3 SUMMARY

The FDA and FSIS of the USDA have coordinated their regulations on nutrition labeling. Regulations that implement the Nutrition Labeling and Education Act (NLEA) of 1990 require nutrition labeling for most foods regulated by the FDA, and FSIS requires the same label on most meat and poultry products. The regulations define the format for the nutrition information, and give the rules and methods to report specific

information. The FDA and FSIS have described the sample collection procedures, the method of analysis to be used, and the nutrient levels required to ensure compliance with nutrition labeling regulations. The FDA and FSIS allow specific nutrient content claims and FDA allows health claims on the nutrition label. The NLEA provides for national uniformity in nutrition labeling, by preempting any existing state regulations, and authorizes to states certain enforcement power. The goal of current nutrition labeling regulations is to provide consumers in all states with nutrition information on food in their diets consistent with their dietary concerns.

3.4 STUDY QUESTIONS

1. Utilize the data in the table below that you obtained on the nutrient content of your cereal product (actual amount per serving) to help develop a nutrition label that meets FDA requirements under the NLEA. Use appropriate rounding rules to complete the blank columns. Can you make a "low fat" claim? Explain your answer. Could you use the term "healthy" on the label? If you wanted to report the

protein content as a percent of the Daily Value, what would you need to do?

	Actual Amount Per Serving[1]	Amount Per Serving Reported on Label	% Daily Value[2] Reported on Label
Calories	19.2		—
Calories from Fat	1.1		—
Total Fat	1.1 g		
Saturated Fat	0 g		
Cholesterol	0 g		
Sodium	268 mg		
Potassium	217 mg		
Total Carbohydrate	44.3 g		
Dietary Fiber	3.8 g		
Sugars	20.2 g		—
Protein	3.7 g		—

[1]Serving size is 1 cup (55 g).
[2]Percent Daily Value based on a 2000 calorie diet.

2. The FDA and FSIS of the USDA have very similar regulations for nutrition labeling, with differences due primarily to the inherent difference in the food products they regulate. Identify the differences in regulations between FDA and FSIS regarding nutrient content claims and health claims that support this generalization.

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16. USDA. February 1993. *Food Safety Inspection Service (FSIS) Manual on Use of Data Bases for Nutrition Labeling. FSIS Guidelines for Effective use of Data Bases to Develop Nutrient Declarations for Nutrition Labeling of Meat and Poultry Products*. Food Safety and Inspection Service, U.S. Department of Agriculture, Washington, DC.

3.6 RELEVANT INTERNET ADDRESSES

Code of Federal Regulations
http://www.access.gpo.gov/nara/cfr/cfr-table-search.html

Food and Drug Administration
http://www.fda.gov
Center for Food Safety & Applied Nutrition
http://vm.cfsan.fda.gov/list.html
Food Labeling Guide
http://vm.cfsan.fda.gov/~dms/flg-toc.html
Food Labeling and Nutrition
http://vm.cfsan.fda.gov/label.html

U.S. Department of Agriculture
http://www.usda.gov

Food Safety and Inspection Service

http://www.usda.gov/fsis
Nutrient Data Laboratory (USDA Handbook No. 8)
    http://www.nal.usda.gov/fnic/foodcomp

ACKNOWLEDGMENTS

Evaluation of Analytical Data

J. Scott Smith

4.1 INTRODUCTION

The field of food analysis, or any type of analysis, involves a considerable amount of time learning principles, methods, and instrument operations and perfecting various techniques. Although these areas are extremely important, much of our effort would be for naught if there were not some way for us to evaluate the data obtained from the various analytical assays. Several mathematical treatments are available that provide an idea of how well a particular assay was performed or how well we can reproduce an experiment. Fortunately, the statistics are not too involved and apply to most analytical determinations.

The focus in this chapter is primarily on how to evaluate replicate analyses of the same sample for accuracy and precision. In addition, considerable attention is given to the determination of best line fits for standard curve data. Keep in mind as you read and work through this chapter that there is a vast array of computer software to perform most types of data evaluation and calculations/plots.

Proper sampling and sample size are not covered in this chapter. Readers should refer to Chapter 5 (especially section 5.3.4.5) for sampling in general and statistical approaches to determine the appropriate sample size, and to Chapter 20, section 20.2.2 for mycotoxin sampling.

4.2 MEASURES OF CENTRAL TENDENCY

To increase accuracy and precision, as well as to evaluate these parameters, the analysis of a sample is usually performed (repeated) several times. At least three assays are typically performed, though often the number can be much higher. Because we are not sure which value is closest to the true value, we determine the mean (or average) using all the values obtained and report the results of the mean. The mean is designated by the symbol $\bar{x}$ and calculated according to the equation below.

$$\bar{x} = \frac{x_1 + x_2 + x_3 + \ldots + x_n}{n} = \frac{\Sigma x_i}{n} \qquad [1]$$

where:

$\bar{x}$ = mean
$x_1, x_2,$ etc. = individually measured values (x_i)
n = number of measurements

For example, suppose we measured a sample of uncooked hamburger for percent moisture content four times and obtained the following results: 64.53%, 64.45%, 65.10%, and 64.78%.

$$\bar{x} = \frac{64.53 + 64.45 + 65.10 + 64.78}{4} = 64.72\% \qquad [2]$$

Thus, the result would be reported as 64.72% moisture. When we report the mean value, we are indicating that this is the best experimental estimate of the value. We are not saying anything about how accurate or true this value is. Some of the individual values may be closer to the true value, but there is no way to make that determination, so we report only the mean.

Another determination that can be used is the median, which is the midpoint or middle number within a group of numbers. Basically, half of the experimental values will be less than the median and half will be greater. The median is not used often, because the mean is such a superior experimental estimator.

4.3 RELIABILITY OF ANALYSIS

Returning to our previous example, recall that we obtained a mean value for moisture. However, we did not have any indication of how repeatable the tests were or how close our results were to the true value. The next several sections will deal with these questions and some of the relatively simple ways to calculate the answers.

4.3.1 Accuracy and Precision

One of the most confusing aspects of data analysis for students is grasping the concepts of accuracy and precision. These terms are commonly used interchangeably in society, which only adds to this confusion. If we consider the purpose of the analysis, then these terms become much clearer. If we look at our experiments, we know that the first data obtained are the individual results and a mean value (0). The next questions should be: How close were our individual measurements? and How close were they to the true value? Both questions involve accuracy and precision. Now, let us turn our attention to these terms.

Accuracy refers to how close a particular measure is to the true or correct value. In the moisture analysis for hamburger, recall that we obtained a mean of 64.72%. Let us say the true moisture value was actually 65.05%. By comparing these two numbers, you could probably make a guess that your results were fairly accurate because they were close to the correct value. (The calculations of accuracy will be discussed later.)

The problem in determining accuracy is that most of the time we are not sure what the true value is. For certain types of materials we can purchase known samples from, for example, the National Institute of Standards and Technology, and check our assays against these samples. Only then can we have an indication of the accuracy of the testing procedures. Another approach is to compare our results with those of other labs to determine how well they agree, assuming the other labs are accurate.

A term that is much easier to deal with and determine is precision. This parameter is a measure of how reproducible or how close replicate measurements become. If repetitive testing yields similar results, then we would say the precision of that test was good. From a true statistical view, the precision often is called error, when we are actually looking at experimental variation. So, the concepts of precision, error, and variation are closely related.

The difference between precision and accuracy can be illustrated best with Fig. 4-1. Imagine shooting a rifle at a target that represents experimental values. The bull's eye would be the true value, and where the bullets hit would represent the individual experimental values. As you can see in Fig. 4-1a, the values can be tightly spaced (good precision) and close to the bull's eye (good accuracy); or, in some cases, there can be situations with good precision but poor accuracy (Fig. 4-1b). The worst situation, as illustrated in Fig. 4-1d, is when both the accuracy and precision are poor. In this case, because of errors or variation in the determination, interpretation of the results becomes very difficult. Later, the practical aspects of the various types of error will be discussed.

When evaluating data, several tests are used commonly to give some appreciation of how much the experimental values would vary if we were to repeat the test (indicators of precision). An easy way to look at the variation or scattering is to report the range of the experimental values. The range is simply the difference between the largest and smallest observation. This measurement is not too useful and thus is seldom used in evaluating data.

Probably the best and most commonly used statistical evaluation of the precision of analytical data is the standard deviation. The standard deviation measures the spread of the experimental values and gives a good indication of how close each of the values are to each other. When evaluating the standard deviation, one has to remember that we are never able to analyze the

entire food product. That would be difficult, if not impossible, and very time consuming. Thus, the calculations we use are only estimates of the unknown true value.

If we have many samples, then the standard deviation is designated by the Greek letter sigma (σ). It is calculated according to Equation [3], assuming all the food product was evaluated (which would be an infinite amount of assays).

$$\sigma = \sqrt{\frac{\Sigma(x_i - \mu)^2}{n}} \qquad [3]$$

where:

σ = standard deviation
x_i = individual sample values
μ = true mean
n = total population of samples

Because we do not know the value for the true mean, the equation becomes somewhat simplified so that we can use it with real data. In this case, we now call the σ term the standard deviation of the sample and designate is by SD or σ. It is determined according to the calculation in Equation [4] where $\bar{x}$ replaces the true mean term μ, and n represents the number of samples.

$$SD = \sqrt{\frac{\Sigma(x_i - \bar{x})^2}{n}} \qquad [4]$$

If the number of replicate determinations is small (about 30 or less), which is common with most assays, the n is replaced by the $n - 1$ term, and Equation [5] is used. Unless you know otherwise, Equation [5] is always used in calculating the standard deviation of a group of assays.

$$SD = \sqrt{\frac{\Sigma(x_i - \bar{x})^2}{n - 1}} \qquad [5]$$

Depending on which of the equations above is used, the standard deviation may be reported as SD$_n$ or

a   b   c   d
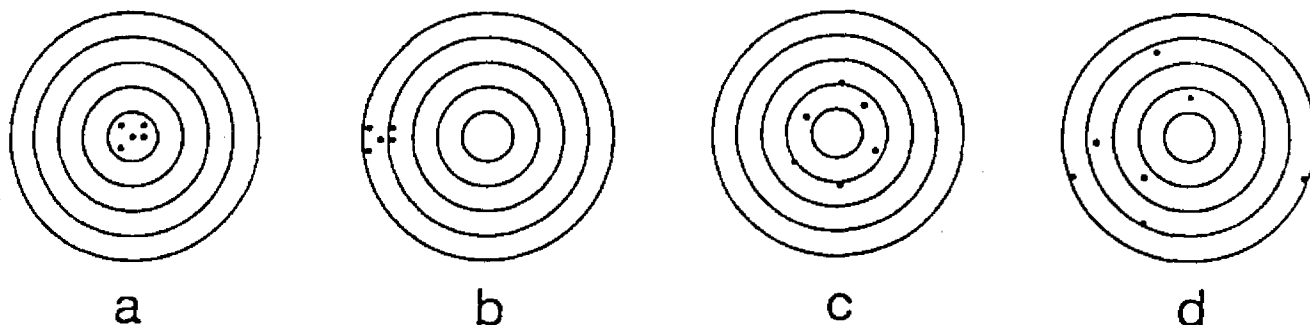
4-1 Comparison of accuracy and precision: (a) good accuracy and good precision, (b) good precision and poor accuracy, (c) good accuracy and poor precision, and (d) poor accuracy and poor precision.
figure

table 4-1 — Determination of the Standard Deviation of Percent Moisture in Uncooked Hamburger

Measurement	Observed % Moisture	Deviation from the Mean $(x_i - \bar{x})$	$(x_i - \bar{x})^2$
1	64.53	−0.19	0.0361
2	64.45	−0.27	0.0729
3	65.10	+0.38	0.1444
4	64.78	+0.06	0.0036
	$\Sigma x_i = 258.86$		$\Sigma(x_i - \bar{x})^2 = 0.257$

$$\bar{x} = \frac{\Sigma x_i}{n} = \frac{258.86}{4} = 64.72$$

$$SD = \sqrt{\frac{\Sigma(x_i - \bar{x})^2}{n-1}} = \sqrt{\frac{0.257}{3}} = 0.2927$$

figure 4-2

A normal distribution curve for a population or a group of analyses.

σ_n and SD_{n-1} or σ_{n-1}. (Different brands of software and scientific calculators sometimes use different labels for the keys, so you must be careful.) Table 4-1 shows an example of the determination of standard deviation. The sample results would be reported to average 64.72% moisture with a standard deviation of 0.293.

Once we have a mean and standard deviation, we next must determine how to interpret these numbers. One easy way to get a feel for the standard deviation is to calculate what is called the coefficient of variation (CV), also known as the relative standard deviation. This calculation is shown below for our example of the moisture determination of uncooked hamburger.

$$\text{Coefficient of Variation (CV)} = \frac{SD}{\bar{x}} \times 100\% \quad [6]$$

$$CV = \frac{0.293}{64.72} \times 100\% = 0.453\% \quad [7]$$

The coefficient of variation tells us that our standard deviation is only 0.453% as large as the mean. For our example, that number is small, which indicates a high level of precision or reproducibility of the replicates. As a rule, a CV below 5% is considered acceptable, although it depends on the type of analysis.

Another way to evaluate the meaning of the standard deviation is to examine its origin in statistical theory. Many populations (in our case, sample values or means) that exist in nature are said to have a normal distribution. If we were to measure an infinite number of samples, we would get a distribution similar to that represented by Fig. 4-2. In a population with a normal distribution, 68% of those values would be within ±1 standard deviation from the mean; 95% would be within ±2 standard deviations; and 99.7% would be within ±3 standard deviations. In other words, there is a probability of less than 1% that a sample in a popula-

tion would fall outside ±3 standard deviations from the mean value.

Another way of understanding the normal distribution curve is to realize that the probability of finding the true mean is within certain confidence intervals as defined by the standard deviation. For large numbers of samples, we can determine the confidence limit or interval around the mean using the statistical parameter called the Z value. We do this calculation by first looking up the Z value from statistical tables once we have decided the desired degree of certainty. Some Z values are listed in Table 4-2.

The confidence limit (or interval) for our moisture data, assuming a 95% probability, is calculated according to Equation [8]. Since this calculation is not valid for small numbers, assume we ran 25 samples instead of four.

Confidence Interval (CI) =

$$\bar{x} \pm Z \text{ value} \times \frac{\text{standard deviation (SD)}}{\sqrt{n}} \quad [8]$$

$$\text{Confidence Interval (at 95\%)} = 64.72 \pm 1.96 \times \frac{0.2927}{\sqrt{25}}$$

$$= 64.72 \pm 0.115\% \quad [9]$$

table 4-2 — Values for Z for Checking Both Upper and Lower Levels

Degree of Certainty (Confidence)	Z Value
80%	1.29
90%	1.64
95%	1.96
99%	2.58
99.9%	3.29

4-3 table
Values of t for Various Levels of Probability[1]

Degrees of Freedom $(n - 1)$	Levels of Certainty		
	95%	99%	99.9%
1	12.7	63.7	636
2	4.30	9.93	31.60
3	3.18	5.84	12.9
4	2.78	4.60	8.61
5	2.57	4.03	6.86
6	2.45	3.71	5.96
7	2.36	3.50	5.40
8	2.31	3.56	5.04
9	2.26	3.25	4.78
10	2.23	3.17	4.59

[1]More extensive t-tables can be found in statistics books.

Because our example had only four values for the moisture levels, the confidence interval should be calculated using statistical t tables. In this case, we have to look up the t-value from Table 4-3 based on the degree of freedom, which is the sample size minus one $(n - 1)$, and the desired level of confidence.

The calculation for our moisture example with four samples (n) and 3 degrees of freedom $(n - 1)$ is given below.

Confidence Interval =

$$\bar{x} \pm t \text{ value} \times \frac{\text{standard deviation (SD)}}{\sqrt{n}} \quad [10]$$

$$\text{Confidence Interval (at 95\%)} = 64.72 \pm 3.18 \times \frac{0.2927}{\sqrt{4}}$$

$$= 64.72 \pm 0.465\% \quad [11]$$

To interpret this number, we can say that, with 95% confidence, the true mean for our moisture will fall within 64.72 ± 0.465% or between 65.185 and 64.255%.

The expression $SD/\sqrt{n}$ is often reported as the standard error of the mean. It then is left to the reader to calculate the confidence interval based on the desired level of certainty.

Other quick tests of precision used are the relative deviation from the mean and the relative average deviation from the mean. The relative deviation from the mean is useful when only two replicates have been performed. It is calculated according to Equation [12], with values below 2% considered acceptable.

Relative deviation from the mean

$$= \frac{x_i - \bar{x}}{\bar{x}} \times 100 \quad [12]$$

The x_i represents the individual sample value, and $\bar{x}$ is the mean.

If there are several experimental values, then the relative average deviation from the mean becomes a useful indicator of precision. It is calculated similarly to the relative deviation from the mean, except the average deviation is used instead of the individual deviation. It is calculated according to Equation [13].

Relative average deviation from the mean =

$$\frac{\frac{\Sigma |x_i - \bar{x}|}{n}}{\bar{x}} \times 1000 = \text{parts per thousand} \quad [13]$$

Using the moisture values discussed in Table 4-1, the $x_i - \bar{x}$ terms for each determination are −0.19, −0.27, +0.38, +0.06. Thus, the calculation becomes:

$$\text{Rel. Avg. Dev.} = \frac{\frac{0.19 + 0.27 + 0.38 + 0.06}{4}}{64.72} \times 1000$$

$$= \frac{0.225}{64.72} \times 1000$$

$$= 3.47 \text{ parts per thousand} \quad [14]$$

Up to now, our discussions of calculations have involved ways to evaluate precision. If the true value is not known, we can calculate only precision. A low degree of precision would make it difficult to predict a realistic value for the sample.

However, we occasionally may have a sample for which we know the true value and can compare our results with the known value. In this case, we can calculate the error for our test, compare it to the known value, and determine the accuracy. One term that can be calculated is the absolute error, which is simply the difference between the experimental value and the true value.

$$\text{Absolute error} = E_{abs} = x - T \quad [15]$$

where:

x = experimentally determined value
T = true value

The absolute error term can have either a positive or negative value. If the experimentally determined value is from several replicates, then the mean (0) would be substituted for the x term. This is not a good test for error, because the value is not related to the magnitude of the true value. A more useful measurement of error is relative error.

$$\text{Relative error} = E_{rel} = \frac{E_{abs}}{T} = \frac{x - T}{T} \quad [16]$$

The results are reported as a negative or positive value, which represents a fraction of the true value.

If desired, the relative error can be expressed as % relative error by multiplying by 100%. Then the rela-

tionship becomes the following, where x can be either an individual determination or the mean (0) of several determinations.

$$\%E_{rel} = \frac{E_{abs}}{T} \times 100\% = \frac{x - T}{T} \times 100\% \qquad [17]$$

Using the data for the % moisture of uncooked hamburger, suppose the true value of the sample is 65.05%. The % relative error is calculated using our mean value of 64.72% and Equation [17].

$$\%E_{rel} + \frac{x - T}{T} \times 100\%$$

$$= \frac{64.72 - 65.05}{65.05} \times 100\% = -0.507\% \qquad [18]$$

Note that we keep the negative value, which indicates the direction of our error, i.e., our results were 0.507% lower than the true value.

4.3.2 Sources of Errors

As you may recall from our discussions of accuracy and precision, error (variation) can be quite important in analytical determinations. Although we strive to obtain correct results, it is unreasonable to expect an analytical technique to be entirely free of error. The best we can hope for is that the variation is small and, if possible, at least consistent. As long as we know about the error, the analytical method often will be satisfactory. There are several sources of error, which can be classified as: systematic error (determinate), random error (indeterminate); and gross error or blunders. Again, note that error and variation are used interchangeably in this section and essentially have the same meaning for these discussions.

Systematic or determinate error produces results that consistently deviate from the expected value in one direction or the other. As illustrated in Fig. 4-1b, the results are spaced closely together, but they are consistently off the target. Identifying the source of this serious type of error can be difficult and time consuming, because it often involves inaccurate instruments or measuring devices. For example, a pipette that consistently delivers the wrong volume of reagent will produce a high degree of precision yet inaccurate results. Sometimes impure chemicals or the analytical method itself are the cause. Generally, we can overcome systematic errors by proper calibration of instruments, running blank determinations, or using a different analytical method.

Random or indeterminate errors are always present in any analytical measurement. This type of error is due to our natural limitations in measuring a particular system. These errors fluctuate in a random fashion and are essentially unavoidable. For example, reading

an analytical balance, judging the endpoint change in a titration, and using a pipette all contribute to random error. Background instrument noise, which is always present to some extent, is a factor in random error. Both positive and negative errors are equally possible. Although this type of error is difficult to avoid, fortunately it is usually small.

Blunders are easy to eliminate, since they are so obvious. The experimental data are usually scattered, and the results are not close to an expected value. This type of error is a result of using the wrong reagent or instrument or from sloppy technique. Some people have called this type of error the "Monday morning syndrome" error. Fortunately, blunders are easily identified and corrected.

4.3.3 Specificity

Specificity of a particular analytical method means that it detects only the component of interest. Analytical methods can be very specific for a certain food component or, in many cases, can analyze a broad spectrum of components. Quite often, it is desirable for the method to be somewhat broad in its detection. For example, the determination of food lipid (fat) is actually the crude analysis of any compound that is soluble in an organic solvent. Some of these compounds are glycerides, phospholipids, carotenes, and free fatty acids. Since we are not concerned about each individual compound when considering the crude fat content of food, it is desirable that the method be broad in scope. On the other hand, determining the lactose content of ice cream would require a specific method. Because ice cream contains other types of simple sugars, without a specific method we would overestimate the amount of lactose present.

There are no hard rules for what specificity is required. Each situation is different and depends on the desired results and type of assay used. However, it is something to keep in mind as the various analytical techniques are discussed.

4.3.4 Sensitivity and Detection Limit

Although often used interchangeably, the terms sensitivity and detection limit should not be confused. They have different meanings yet are closely related. Sensitivity relates to the magnitude of change of a measuring device (instrument) with changes in compound concentration. It is an indicator of how little change can be made in the unknown material before we notice a difference on a needle gauge or a digital readout. We are all familiar with the process of tuning in a radio station on our stereo and know how, at some point, once the station is tuned in, we can move the dial without disturbing the reception. This is sensitivity. In many

situations, we can adjust the sensitivity of an assay to fit our needs, i.e. whether we desire more or less sensitivity. We even may desire a lower sensitivity so that samples with widely varying concentration can be analyzed at the same time.

Detection limit, in contrast to sensitivity, is the lowest possible increment that we can detect with some degree of confidence (or statistical significance). With every assay, there is a lower limit at which point we are not sure if something is present or not. Obviously, the best choice would be to concentrate the sample so we are not working close to the detection limit. However, this may not be possible, and we may need to know the detection limit so we can work away from that limit.

There are several ways to measure the detection limit, depending on the apparatus that is used. If we are using something like a spectrophotometer, gas chromatograph, or high performance liquid chromatograph (HPLC) the limit of detection often is reached when the signal-to-noise ratio is 3 or greater. In other words, when the sample gives a value that is three times the magnitude of the noise detection, the instrument is at the lowest limit possible. Noise is the random signal fluctuation that occurs with any instrument.

A more general way to define the limit of detection is to approach the problem from a statistical viewpoint, in which the variation between samples is considered. A common mathematical definition of detection limit is given below.

$$X_{LD} = X_{Blk} + (3 \times SD_{Blk})$$ [19]

where:

X_{LD} = the minimum detectable concentration
X_{Blk} = the signal of a blank
SD_{Blk} = the standard deviation of the blank readings

In this equation, the variation of the blank values (or noise, if we are talking about instruments) determines the detection limit. High variability in the blank values decreases the limit of detection.

4.4 CURVE FITTING: REGRESSION ANALYSIS

Curve fitting is a generic term used to describe the relationship and evaluation between two variables. Most scientific fields use curve fitting procedures to evaluate the relationship of two variables. Thus, curve fitting or curvilinear analysis of data is a vast area as evidenced by the volumes of material describing these procedures. In analytical determinations, we are usually concerned with only a small segment of curvilinear analysis, the standard curve or regression line.

A standard curve or calibration curve is used to determine unknown concentrations based on a method

that gives some type of measurable response that is proportional to a known amount of standard. It typically involves making a group of known standards in increasing concentration and then recording the particular measured analytical parameter (e.g., absorbance, area of a chromatography peak, etc.). What results when we graph the paired x and y values is a scatterplot of points that can be joined together to form a straight line relating concentration to observed response. Once we know how the observed values change with concentration, it is fairly easy to estimate the concentration of an unknown by interpolation from the standard curve.

As you read through the next three sections, keep in mind that not all correlations of observed values to standard concentrations are linear (but most are). There are many examples of nonlinear curves, such as antibody binding, toxicity evaluations, and exponential growth and decay. Fortunately, with the vast array of computer software available today, it is relatively easy to analyze any group of data.

4.4.1 Linear Regression

So how do we set up a standard curve once the data have been collected? First a decision must be made regarding onto which axis to plot the paired sets of data. Traditionally the concentration of the standards are represented on the x-axis and the observed readings are on the y-axis. However, this protocol is used for reasons other than convention. The x-axis data are called the dependent variable and assumed to be essentially free of error, while the y-axis data (the independent variable) may have error associated with them. This assumption may not be true because error could be incorporated as the standards are made. With modern day instruments the error can be very small. Although arguments can be made for making the y-axis data concentration, for all practical purposes the end result is essential the same. Unless there are some unusual data, the concentration should be associated with the x-axis and the measured values with the y-axis.

Figure 4-3 illustrates a typical standard curve used in the determination of caffeine in various foods. Caffeine is analyzed readily in foods by using HPLC coupled with an ultraviolet detector set at 272 nm. The area under the caffeine peak at 272 nm is directly proportional to the concentration. When an unknown sample (e.g., coffee) is run on the HPLC, a peak area is obtained that can be related back to the sample using the standard curve.

The plot in Fig. 4-3 shows all the data points and a straight line that appears to pass through most of the points. The line almost passes through the origin, which makes sense because zero concentration should produce no signal at 272 nm. However, the line is not

Conc (x)	Peak Area (y)
5	488
25	2291
50	4795
75	6780
100	9048
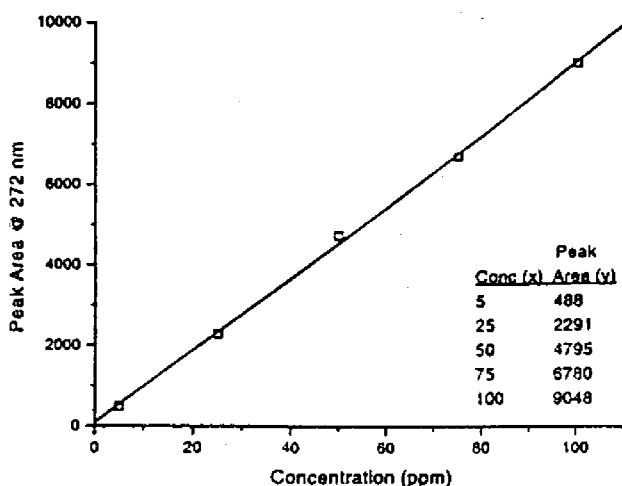
4-3 figure A typical standard curve plot showing the data points and the generated best fit line. The data used to plot the curve are presented on the graph.

perfectly straight (and never is) and does not quite pass through the origin.

To determine the caffeine concentration in a sample that gave an area of say 4000, we could extrapolate to the line and then draw a line down to the x-axis. Following the dashed line to the x-axis (concentration), we can estimate the solution to be at about 42–43 ppm of caffeine.

We can mathematically determine the best fit of the line by using linear regression. Keep in mind the equation for a straight line, which is: $y = ax + b$, where a = slope and b = y-intercept. To determine the slope and y-intercept, the regression equations shown below are used. We determine a and b and thus, for any value of y (measured), we can determine the concentration (x).

$$\text{slope } a = \frac{\Sigma(x_i - \bar{x})(y_i - \bar{y})}{\Sigma(x_i - \bar{x})^2} \quad [20]$$

$$y\text{-intercept } b = \bar{y} - a\bar{x} \quad [21]$$

The x_i and y_i parameters are the individual values, and $\bar{x}$ and $\bar{y}$ are the means of the individual values. Low cost calculators and computer spreadsheet software can readily calculate regression equations so no attempt is made to go through the mathematics in the formulas.

The formulas give what is known as the line of regression of y on x which assumes that the error occurs in the y direction. The regression line represents the average relationship between all the data points and thus is a balanced line. These equations also assume that the straight line fit does not have to go through the origin, which at first does not make much sense. However, there are often background interferences so that even at zero concentration a weak signal

may be observed. In most situations, calculating the origin as going through zero will yield the same results.

Using the data from Fig. 4-3, calculate the concentration of caffeine in the unknown and compare with the graphing method. As you recall, the unknown had an area at 272 nm of 4000. Linear regression analysis of the standard curve data gave the y-intercept (b) as 84.66118 and the slope (a) as 90.07331.

$$y = ax + b \quad [22]$$

or

$$x = \frac{y - b}{a} \quad [23]$$

$$x(\text{conc}) = \frac{4000 - 84.66118}{90.07331} = 43.468 \text{ ppm caffeine} \quad [24]$$

The agreement is fairly close when comparing the calculated value to that estimated from the graph. Using high quality graph paper with many lines could give us a line very close to the calculated one. However, as we will see in the next section, additional information can be obtained about the nature of the line when using computer software or calculators.

4.4.2 Correlation Coefficient

In observing any type of correlation, including linear ones, questions always surface concerning how to draw the line through the data points and how well do the data fit to the straight line. The first thing that should be done with any group of data is to plot it to see if the points fit a straight line. By just looking at the plotted data, it is fairly easy to make a judgment on the linearity of the line. We also can pick out regions on the line where a linear relationship does not exist. The figures below illustrate differences in standard curves; Fig. 4-4a shows a good correlation of the data and Fig. 4-4b shows a poor correlation. In both cases, we can
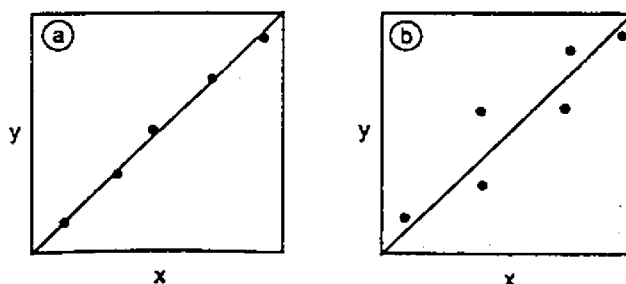
4-4 figure Examples of standard curves showing the relationship between the X and Y variables when there is (a) a high amount of correlation and (b) a lower amount of correlation. Both lines have the same equation.

draw a straight line through the data points. Both curves yield the same straight line but the precision is poorer for the latter.

There are other possibilities when working with standard curves. Figure 4-5a shows a good correlation between x and y but in the negative direction, and Fig. 4-5b illustrates data that have no correlation at all.

The correlation coefficient defines how well the data fit to a straight line. For a standard curve, the ideal situation would be that all data points lie perfectly on a straight line. However, this is never the case, because errors are introduced in making standards and measuring the physical values (observations).

The correlation coefficient and coefficient of determination are defined below. Essentially all spreadsheet and plotting software will calculate the values automatically.

$$\text{correlation coefficient} =$$
$$r = \frac{\Sigma(x_i - \bar{x})(y_i - \bar{y})}{\sqrt{[\Sigma(x_i - \bar{x})^2][\Sigma(y_i - \bar{y})^2]}} \quad [25]$$

For our example of the caffeine standard curve from Fig. 4-3:

$r = 0.99943$ (values are usually reported to at least 4 significant figures)

For standard curves, we want the value of r as close to +1.0000 or −1.000 as possible, because this value is a perfect correlation (perfect straight line). Generally, in analytical work, the r should be 0.9970 or better. (This does not apply to biological studies.)

The coefficient of determination (r^2) is used quite often because it gives a better perception of the straight line even though it does not indicate the direction of the correlation. The r^2 for the example presented above is 0.99886, which represents the proportion of the variance of absorbance (y) that can be attributed to its linear regression on concentration (x). This means that about 0.114% of the straight line variation (1.0000 −

0.99886 = 0.00114 × 100% = 0.114%) does not vary with changes in x and y and, thus is due to indeterminate variation. A small amount of variation is expected normally.

4.4.3 Errors in Regression Lines

While the correlation coefficient tells us something about the error or variation in linear curve fits, it does not always give the complete picture. Also, neither linear regression nor correlation coefficient will indicate that a particular set of data have a linear relationship. They only provide an estimate of the fit assuming the line is a linear one. As indicated before, plotting the data is critical when looking at how the data fit on the curve (actually, a line). One parameter that is used often is the y-residuals, which are simply the differences between the observed values and the calculated or computed values (from the regression line). Advanced computer graphics software can actually plot the residuals for each data point as a function of concentration. However, plotting the residuals is usually not necessary because data that do not fit on the line are usually quite obvious. If the residuals are large for the entire curve, then the entire method needs to be evaluated carefully. However, the presence of one point that is obviously off the line while the rest of the points fit very well probably indicates an improperly made standard.

One way to reduce the amount of error is to include more replicates of the data such as repeating the observations with a new set of standards. The replicate x and y values can be entered into the calculator or spreadsheet as separate points for the regression and coefficient determinations. Another, probably more desirable, option is to expand the concentrations at which the readings are taken. Collecting observations at more data points (concentrations) will produce a better standard curve. However, increasing the data beyond seven or eight points usually is not beneficial.

Plotting confidence intervals, or bands or limits on the standard curve along with the regression line is another way to gain insight into the reliability of the standard curve. Confidence bands define the statistical uncertainty of the regression line at a chosen probability (such as 95%) using the t-statistic and the calculated standard deviation of the fit. In some aspects, the confidence bands on the standard curve are similar to the confidence interval discussed in section 4.3.1. However, in this case we are looking at a line rather than a confidence interval around a mean. Figure 4-6 shows the caffeine data from the standard curve presented before, except some of the numbers have been modified to enhance the confidence bands. The confidence bands (dashed lines) consist of both an upper limit and
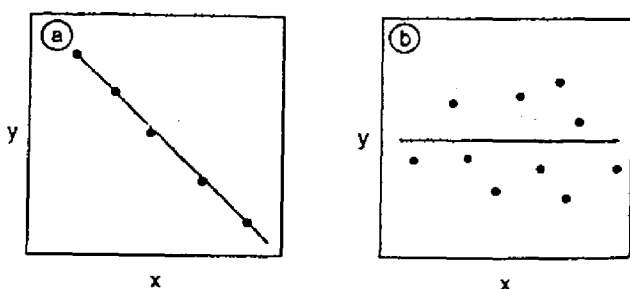
4-5 figure Examples of standard curves showing the relationship between the x and y variables when there is (a) a high amount of negative correlation and (b) no correlation between x and y values.

Linear Fit Data
y = 80.388 (x) + 244.29
r = 0.98183

Peak Conc (x)	Area (y)
5	310
25	2100
50	4795
75	6950
100	7585
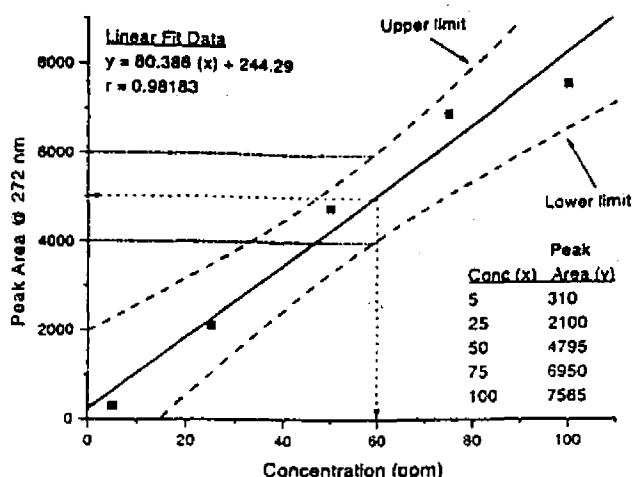
4-6 A standard curve graph showing the confidence bands. The data used to plot the graph are presented on the graph as are the equation of the line and the correlation coefficient.

4-7 A standard curve plot showing possible deviations in the curve in the upper and lower limits.

a lower limit that define the variation of the y-axis value. The upper and lower bands are narrowest at the center of the curve and get wider as the curve moves to the higher or lower standard concentrations.

Looking at Fig. 4-6 again, note that the confidence bands show what amount of variation we expect in a peak area at a particular concentration. At 60 ppm concentration, by going up from the x-axis to the bands and extrapolating to the y-axis, we see that with our data the 95% confidence interval of the observed peak area will be 4000 to 6000. In this case, the variation is large and would not be acceptable as a standard curve, and is presented here only for illustration purposes.

Error bars also can be used to show the variation of y at each data point. Several types of error or variation statistics can be used such as standard error, standard deviation, or percentage of data (i.e., 5%). Any of these methods give a visual indication of experimental variation.

Unfortunately, confidence bands and error bars are not used often because the mathematics is difficult and most calculators and spreadsheet software do not have that capability. Construction of confidence bands requires the use of advanced graphics software such as Microcal's Origin[3].

Even with good standard curve data, problems can arise if the standard curve is not used properly. One common mistake is to extrapolate beyond the data points used to construct the curve. Figure 4-7 illustrates some of the possible problems that might occur when extrapolation is used. As shown in Fig. 4-7, the curve or line may not be linear outside the area where the data were collected. This can occur in the region close to the origin or especially at the higher concentration level.
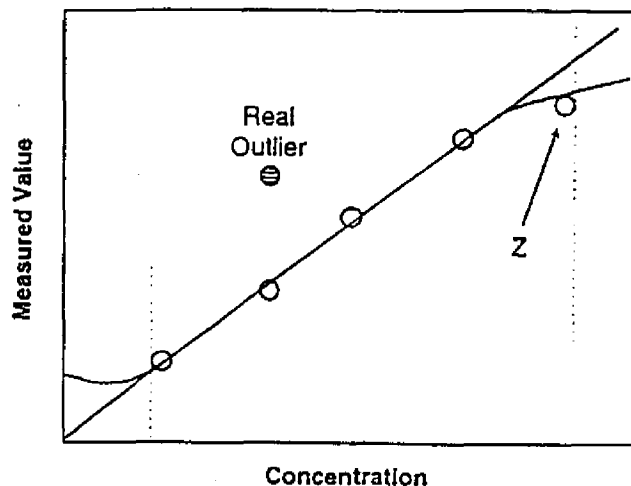
Usually a standard curve will go through the origin, but in some situations it may actually tail off as zero concentration is approached. At the other end of the curve, at higher concentrations, it is fairly common for a plateau to be reached where the measured parameter does not change much with an increase in concentration. Care must be used at the upper limit of the curve to ensure that data for unknowns are not collected outside of the curve standards. Point Z on Fig. 4-7 should be evaluated carefully to determine if the point is an outlier or if the curve is actually tailing off. Collecting several sets of data at even higher concentrations should clarify this. Regardless, the unknowns should be measured only in the region of the curve that is linear.

4.5 REPORTING RESULTS

In dealing with experimental results, we always are confronted with reporting data in a way that indicates the sensitivity and precision of the assay. Ideally, we do not want to overstate or understate the sensitivity of the assay, and thus strive to report a meaningful value, be it a mean, standard deviation, or some other number. The next three sections discuss how we can evaluate experimental values so as to be precise when reporting results.

4.5.1 Significant Figures

The term significant figure is used rather loosely to describe some judgment of the number of reportable digits in a result. Often, the judgment is not soundly based, and meaningful digits are lost or meaningless digits are retained. Exact rules are provided below to

help determine the number of significant figures to report. However, it is important to keep some flexibility when working with significant figures.

Proper use of significant figures is meant to give an indication of the sensitivity and reliability of the analytical method. Thus, reported values should contain only significant figures. A value is made up of significant figures when it contains all digits known to be true and one last digit that is in doubt. For example, a value reported as 64.72 contains four significant figures, of which three digits are certain (64.7) and the last digit is uncertain. Thus, the 2 is somewhat uncertain and could be either 1 or 3. As a rule, numbers that are presented in a value represent the significant figures, regardless of the position of any decimal points. This also is true for values containing zeros, provided they are bounded on either side by a number. For example, 64.72, 6.472, 0.6472, and 6.407 all contain four significant figures. Note that the zero to the left of the decimal point is used only to indicate that there are no numbers above 1. We could have reported the value as .6472, but using the zero is better, since we know that a number was not inadvertently left off our value.

Special considerations are necessary for zeros that may or may not be significant.

1. Zeros after a decimal point are always significant figures. For example, 64.720 and 64.700 both contain five significant figures.
2. Zeros before a decimal point with no other preceding digits are not significant. As indicated before, 0.6472 contains four significant figures.
3. Zeros after a decimal point are not significant if there are no digits before the decimal point. For example, 0.0072 has no digits before the decimal point; thus, this value contains two significant figures. In contrast, the value 1.0072 contains five significant figures.
4. Final zeros in a number are not significant unless indicated otherwise. Thus, the value 7000 contains only one significant figure. However, adding a decimal point and another zero give the number 7000.0, which has five significant figures.

A good way to measure the significance of zeros, if the above rules become confusing, is to convert the number to the exponential form. If the zeros can be omitted, then they are not significant. For example, 7000 expressed in exponential form is 7×10^3 and contains one significant figure. With 7000.0, the zeros are retained and the number becomes 7.0000×10^3. If we were to convert 0.007 to exponent form, the value is 7×10^{-3}, and only one significant figure is indicated. As a rule, determining significant figures in arithmetic operations is dictated by the value having the least number of significant figures.

The easiest way to avoid any confusion is to perform all the calculations and then round off the final answer to the appropriate digits. For example, $36.54 \times 238 \times 1.1 = 9566.172$, and because 1.1 contains only two significant figures, the answer would be reported as 9600 (remember, the two zeros are not significant). This method works fine for most calculations, except when adding or subtracting numbers containing decimals. In those cases, the number of significant figures in the final value is determined by the numbers that follow the decimal point. Thus, adding $7.45 + 8.725 = 16.175$; because 7.45 has only two numbers after the decimal point, the sum is rounded to 16.18. Likewise, $433.8 - 32.66$ gives 401.14, which rounds off to 401.1.

A word of caution is warranted when using the simple rule stated above, for there is a tendency to underestimate the significant figures in the final answer. For example, take the situation in which we determined the caffeine in an unknown solution to be 43.5 ppm (see Equation [24]). We had to dilute the sample 50-fold using a volumetric flask in order to fit the unknown within the range of our method. To calculate the caffeine in the original sample, we multiply our result by 50 or $43.5 \, \mu g/ml \times 50 = 2175 \, \mu g/ml$ in the unknown. Based on our rule above, we then would round the number to one significant figure (because 50 contains one significant figure) and report the value as 2000. However, doing this actually underestimates the sensitivity of our procedure, because we ignore the accuracy of the volumetric flask used for the dilution. A Class-A volumetric flask has a tolerance of 0.05 ml; thus, a more reasonable way to express the dilution factor would be 50.0 instead of 50. We now have increased the significant figures in the answer by two, and the value becomes 2180 mg/ml.

As you can see, an awareness of significant figures and how they are adopted requires close inspection. The guidelines can be helpful but they do not always work, unless each individual value or number is closely inspected.

4.5.2 Rounding Off Numbers

Rounding off numbers is an important and necessary operation in all analytical areas. However, premature or incorrect rounding off can produce serious errors in the final results. It usually is desirable to carry extra numbers during calculations and perform the rounding off on the final answers.

Rounding off procedures are fairly straightforward and commonly used by most everyone. Even the Internal Revenue Service allows taxpayers to round off fractions of a dollar to the whole dollar when filling out income tax forms. However, analytical data require a little more accuracy than the IRS, and thus the rules are slightly different.

The basic rules of rounding off are listed below:

1. If the figure following those numbers to be retained is less than 5, the figure is dropped and the retained numbers are kept unchanged. For example, 64.722 is rounded off to 64.72.
2. If the figure following those numbers to be retained is greater than 5, the figure is dropped, and the last retained number is increased by 1. For example, 64.727 is rounded off to 64.73.
3. If the number following those to be retained is a 5, and there are no figures other than zeros beyond the 5, the figure is dropped and the last retained figure is increased by 1 if it is an odd number, or it is kept unchanged if it is an even number. For example, 64.725 is rounded off to 64.72 and 65.705 is rounded off to 64.70, whereas 64.715 is rounded off to 64.72.

A simplified version of rule 3 above is to increase by 1 if the 5 is followed by numbers other than zeros and ignore the even–odd method. If the 5 is followed by zeros, then it is simply dropped. For example, 64.715 would round off to 64.71, whereas 64.715001 would round off to 64.72.

Remember, it is best to round off after performing any mathematical operations.

4.5.3 Rejecting Data

Inevitably, during the course of working with experimental data we will come across a value that does not match the others. Can you reject that value, and thus not use it in calculating the final reported results?

The answer is "sometimes," but only after careful consideration. If you are routinely rejecting data to help make your assay look better, then you are misrepresenting the results and the precision of the assay. If the bad value resulted from an identifiable mistake in that particular test, then it is probably safe to drop the value. Again, caution is advised, because you may be rejecting a value that is closer to the true value than some of the other values.

Consistently poor accuracy or precision indicates that an improper technique or incorrect reagent was used or that the test was not very good. It is best to make changes in the procedure or change methods rather than try to figure out ways to eliminate undesirable values.

There are several tests for rejecting an aberrant value. One of these tests, the Q-Test, is commonly used. In this test, a Q-value is calculated as shown below and compared to values in a table. If the calculated value is larger than the table value, then the questionable measurement can be rejected at the 90% confidence level.

$$Q\text{-value} = \frac{x_2 - x_1}{W} \qquad [26]$$

where:

x_1 = the questionable value
x_2 = the next closest value to x_1
W = the total spread of all values; obtained by subtracting the lowest value from the highest value

Table 4-4 provides the rejection Q-values for a 90% confidence level.

The example below shows how the test is used for the moisture level of uncooked hamburger for which four replicates were performed giving values of 64.53, 64.45, 64.78, and 55.31. The 55.31 value looks as if it is too low compared to the other results. Can that value be rejected? For our example, x_1 = the questionable value = 55.31, and x_2 is the closest neighbor to x_1 (which is 64.45). The spread (W) is the high value minus the low measurement, which is 64.78 – 55.31.

$$Q\text{-value} = \frac{64.45 - 55.31}{64.78 - 55.31} = \frac{9.14}{9.47} = 0.97 \qquad [27]$$

From Table 4-4, we see that the calculated Q-value must be greater than 0.76 to reject the data. Thus, we make the decision to reject the 55.31% moisture value and do not use it in calculating the mean.

4.6 SUMMARY

This chapter focused on the basic mathematical treatment that most likely will be used in evaluating a group of data. For example, it should be almost second nature to determine a mean, standard deviation, and coefficient of variation when evaluating replicate analyses of an individual sample. In evaluating linear standard curves, best line fits always should be deter-

4-4

table   Q-Values for the Rejection of Results

Number of Observations	Q of Rejection (90% level)
3	0.94
4	0.76
5	0.64
6	0.56
7	0.51
8	0.47
9	0.44
10	0.41

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mined along with the indicators of the degree of linearity (correlation coefficient or coefficient of determination). Fortunately, most computer spreadsheet and graphics software will readily perform the calculations for you. Guidelines are available to enable one to report analytical results in a way that tells something about the sensitivity and confidence of a particular test. These include the proper use of significant figures, rules for rounding off numbers, and use of the Q-test to reject grossly aberrant individual values.

4.7 STUDY QUESTIONS

1. Method A to quantitate a particular food component was reported to be more specific and accurate than method B, but method A had lower precision. Explain what this means.

2. You are considering adopting a new analytical method in your lab to measure moisture content of cereal products. How would you determine the precision of the new method and compare it to the old method? Include any equations to be used for any needed calculations.

3. Differentiate "standard deviation" from "coefficient of variation," "standard error of the mean," and "confidence interval."

4. Differentiate the terms "absolute error" versus "relative error." Which is more useful? Why?

5. For each of the errors described below in performing an analytical procedure, classify the error as random error, systematic error, or blunder, and describe a way to overcome the error.

   a. Automatic pipettor consistently delivered 0.96 ml rather than 1.00 ml.

   b. Substrate was not added to one tube in an enzyme assay.

6. Differentiate the terms "sensitivity" and "detection limit."

7. The correlation coefficient for standard curve A is reported as 0.9970. The coefficient of determination for standard curve B is reported as 0.9950. In which case do the data better fit a straight line?

4.8 PRACTICE PROBLEMS

1. How many significant figures are in the following numbers: 0.0025, 4.50, 5.607?

2. What is the correct answer for the following calculation expressed in the proper amount of significant figures?

$$\frac{2.43 \times 0.01672}{1.83215} =$$

3. Given the following data on dry matter (88.62, 88.74, 89.20, 82.20), determine the mean, standard deviation, and coefficient of variation. Is the precision for this set of data acceptable? Can you reject the value 82.20 since it seems to be different than the others? What is the 95% confidence level you would expect your values to fall within if the test

were repeated? If the true value for dry matter is 89.40, what is the % relative error?

4. Compare the two groups of standard curve data below for sodium determination by atomic emission spectroscopy. Draw the standard curves using graph paper or a computer software program. Which group of data provides a better standard curve? Note that the absorbance of the emitted radiation at 589 nm increases proportionally to sodium concentration. Calculate the amount of sodium in a sample with a value of 0.555 for emission at 589 nm. Use both standard curve groups and compare the results.

Group A—Sodium Standard Curve

Sodium Concentration (µg/ml)	Emission at 589 nm
1.00	0.050
3.00	0.140
5.00	0.242
10.0	0.521
20.0	0.998

Group B—Sodium Standard Curve

Sodium Concentration (µg/ml)	Emission at 589 nm
1.00	0.060
3.00	0.113
5.00	0.221
10.0	0.592
20.0	0.917

Answers:

1. 2, 3, 4. 2. 0.0222. 3. mean = 87.19, SD_{n-1} = 3.34, CV = 3.83%; thus the precision is acceptable. Q_{calc} = 0.92; therefore the value 82.20 can be rejected. CI = 87.19 ± 5.31. %E_{rel} = −2.47%. 4. Group A is the better std. curve (group A, r^2 = 0.9990; group B, r^2 = 0.9708). Sodium in the sample using group A std. curve = 11.1 µg/ml; with group B std. curve = 11.5 µg/mL

4.9 RESOURCE MATERIALS

1. McCormick, D., and Roach, A. 1987. *Measurement, Statistics and Computation.* John Wiley & Sons, New York. This is a very good book covering a wide variety of topics of interest to an analytical chemist. The best feature of this book is that it covers the topics at an introductory level yet with sufficient detail and examples.

2. Miller, J.C., and Miller, J.N. 1988. (Reprinted with corrections 1989.) *Statistics for Analytical Chemistry,* 2nd ed. Ellis Horwood Ltd., distributed by John Wiley & Sons, New York. (Also available from Aldrich Chemical Co., Milwaukee, WI.) This is another excellent introductory text for beginner analytical chemists. It contains a fair amount of detail, sufficient for most analytical statistics, yet works through the material starting at a basic introductory level. The authors also discuss the Q-test used for rejecting data. Unfortunately this book is currently out of print.

3. Skoog, D.A., and West, D.M. 1982. *Fundamentals of Ana-*

lytical Chemistry, 4th ed. Holt-Saunders, New York. Chapter 3 does an excellent job of covering most of the statistics needed by an analytical chemist in an easy-to-read style.

4. Willard, H.H., Merritt, L.L., Jr., Dean, J.A., and Settle, F.A., Jr., 1988. *Instrumental Methods of Analysis*, 7th ed. Wadsworth Publishing, Belmon, CA. This gives a rigorous treatment of instrumentation and has a very useful chapter (Chapter 2, Measurements, Signals and Data) on types of error generated by instruments.

Sampling and Sample Preparation

Andrew Proctor and Jean-François Meullenet

5.1 INTRODUCTION

To control food quality and acceptance within satisfactory limits, it is important to monitor the vital characteristics of raw products, ingredients, and processed foods. This could be done by evaluating all foods or ingredients from a particular lot, which is feasible if the analytical technique is rapid and nondestructive. However, it is usually more practical to select a portion of the total product volume and assume the quality of the selected portion is typical of the whole lot.

Obtaining a portion, or sample, that is representative of the whole is referred to as sampling, and the total quantity from which a sample is obtained is called the population. Adequate sampling technique helps to ensure that sample quality measurements are an accurate and precise estimate of the quality of the population. By sampling only a fraction of the population, a quality estimate can be obtained more quickly and with less expense and personnel time than if the total population were measured. The sample is only an estimate of the true value of the population, but with proper sampling technique it can be a very accurate estimate. Sampling procedures and their selection are discussed in sections 5.2 and 5.3.

A laboratory sample for analysis can be of any size or quantity (1). Factors affecting the sample size and associated problems are discussed in sections 5.3 and 5.4, while preparation of laboratory samples for testing is described in section 5.4.

As you read each section of the chapter, consider application of the information to some specific examples of sampling needs in the food industry: sampling for nutrition labeling (see Study Question 7 in this Chapter), pesticide analysis (see also Chapter 20, section 20.1.3), mycotoxin analysis (see also Chapter 20, section 20.2.2), extraneous matter (see also Chapter 23), or rheological properties (see also Chapter 34). To consider sample collection and preparation for these and other applications subject to government regulations, you are referred also to the sample collection section of compliance procedures established by the Food and Drug Administration (FDA) and Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA) (see Chapter 3, section 3.2.3.1).

It should be noted that sampling terminology and procedures used may vary between companies and between specific applications. However, the principles described in this chapter are intended to provide a basis for understanding, developing, and evaluating sampling plans and sample handling procedures for specific applications encountered.

5.2 SELECTION OF SAMPLING PROCEDURES

5.2.1 General Information

It is important to clearly define the population that is to be sampled. The population may vary in size from a production lot, a day's production, to the contents of a warehouse. Extrapolating information obtained from a sample of a production lot to the population of the lot can be done accurately, but conclusions cannot be drawn from data describing larger populations, such as the whole warehouse.

Populations may be finite, such as the size of a lot, or infinite, such as in the number of temperature observations made of a lot over time (2). For finite populations, sampling provides an estimate of lot quality. In contrast, sampling from infinite populations provides information about a process. Regardless of the population type, i.e., finite or infinite, the data obtained from sampling are compared to a range of acceptable values to ensure the population sampled is within specifications.

Data obtained from an analytical technique are the result of a stepwise procedure from sampling, to sample preparation, laboratory analysis, data processing, and data interpretation. There is a potential for error at each step and the uncertainty, or reliability, of the final result depends on the cumulative errors at each stage (3, 4). Variance is an estimate of the uncertainty. The total variance of the whole testing procedure is equal to the sum of the variances associated with each step of the sampling procedure and represents the precision of the process. Precision is a measure of the reproducibility of the data. In contrast, accuracy is a measure of how close the data are to the true value. The most efficient way to improve accuracy is to improve the reliability of the step with the greatest variance. Frequently, this is the initial sampling step. The reliability of sampling is dependent more on the sample size than on the population size (2). The larger the sample size the more reliable the sampling. However, sample size is limited by time, cost, sampling methods, and the logistics of sample handling, analysis, and data processing.

5.2.2 Sampling Plan

Most sampling is done for a specific purpose (e.g., to determine if the number of defects in the lot is acceptable), and the purpose may dictate the nature of the sampling approach. The International Union of Pure and Applied Chemistry (IUPAC) defines a sampling plan as "a predetermined procedure for the selection, withdrawal, preservation, transportation, and prepa-

ration of the portions to be removed from a lot as samples" (1). A sampling plan should be a well organized document that establishes the required procedures for accomplishing the program's objectives. It should address the issues of who, what, where, why, and how. The primary aim of sampling is to obtain a sample, subject to constraints on size, that will satisfy the sampling plan specifications. A sampling plan should be selected on the basis of the sampling objective, the study population, the statistical unit, the sample selection criteria, and the analysis procedures. The two primary objectives of sampling are often to estimate the average value of a characteristic and determine if the average value meets the specifications defined in the sampling plan.

5.2.3 Factors Affecting Choice of Sampling Plan

Each factor affecting the choice of sampling plans (Table 5-1) must be considered in the selection of a plan. When the purpose of the inspection, the nature of the product, the test method, and lot to be sampled are determined, a sampling plan can be developed that will provide the desired information.

5.2.4 Sampling for Attributes or Variables

Sampling plans are designed for examination of either attributes or variables (2). In attribute sampling, sampling is performed to decide on the acceptability of a population based on whether the sample possesses a certain characteristic, for example, *Clostridium botulinum* contamination in canned goods. Attribute sampling provides data that are in dichotomous form, i.e., data for which there exist two possible alternatives, such as present or absent. The statistical distribution of such a sampling plan is hypergeometric, binomial, or Poisson. In the event of a binomial distribution of the data (e.g., presence of *Clostridium botulinum*), the probability of a single occurrence of the event is directly proportional to the size of the sample. Computing binomial probabilities will allow the investigator to make inferences on the overall lot.

In variable sampling, sampling is performed to estimate quantitatively the amount of a substance (e.g., salt) or a characteristic (e.g., color) on a continuous scale. The estimate obtained from the sample is compared with an acceptable value (i.e., previously determined) and the deviation measured. This type of sampling usually produces data that have a normal distribution, such as in the percent fill of a container and total solids of a food sample. In general, variable sampling requires smaller sample size than attribute sampling (2) and each characteristic should be sampled for separately when possible. However, when FDA and FSIS of USDA do sampling for compliance of nutrition labeling, a composite of 12, and of at least six subsamples, respectively, is obtained and used for all nutrients to be analyzed.

There are three basic types of sampling plans: single, double, or multiple (5). Each may be used for evaluation of attributes or variables, or a combination of both. Selection of the plan depends on the expected overall lot quality and sampling costs. Single sampling plans allow accept/reject decisions to be made by inspection of one sample of a specified size. Double sampling plans require the selection of two sample

Table 5-1 Factors that Affect the Choice of Sampling Plans

Factors to Be Considered	Questions
Purpose of the inspection	Is it to accept or reject the lot?
	Is it to measure the average quality of the lot?
	Is it to determine the variability of the product?
Nature of the product	Is it homogeneous or heterogeneous?
	What is the unit size?
	How consistently have past populations met specifications?
	What is the cost of the material being sampled?
Nature of the test method	Is the test critical or minor?
	Will someone become sick or die if the population fails to pass the test?
	Is the test destructive or nondestructive?
	How much does the test cost to complete?
Nature of the population being investigated	Is the lot large but uniform?
	Does the lot consist of smaller, easily identifiable sublots?
	What is the distribution of the units within the population?

Adapted from (1).

sets. However, if the lot is of extremely high or low quality, acceptance or rejection may be determined after evaluation of the first set of samples. However, if the first sample indicates the lot is of intermediate quality, a second sample set is taken. A decision on the acceptability is then based on analysis of data from both sample sets. The cost associated with multiple sampling plans can be reduced by rejecting low-quality lots and accepting high-quality lots quickly. The amount of sampling depends on the overall lot quality. A multiple sampling chart must be developed to relate the cumulative number of defects to the number of samples taken from the lot (Fig. 5-1). The chart consists of two parallel lines: a rejection line and an acceptance line. When the cumulative number of defects lies above the rejection line, the lot should be rejected. When the number of defects falls below the acceptance line, the lot can be accepted. Sampling should continue until the number of defects crosses the acceptance or the rejection line.

The choice of a sampling plan is an important consideration, especially when monitoring food safety by measurement of fungal toxins, named mycotoxins, in food systems. Mycotoxins are distributed broadly and randomly within a population and a normal distribution cannot be assumed (1). Such distribution requires combination of many randomly selected portions to obtain a reasonable estimate of mycotoxin levels. Methods of analysis that are extremely precise are not
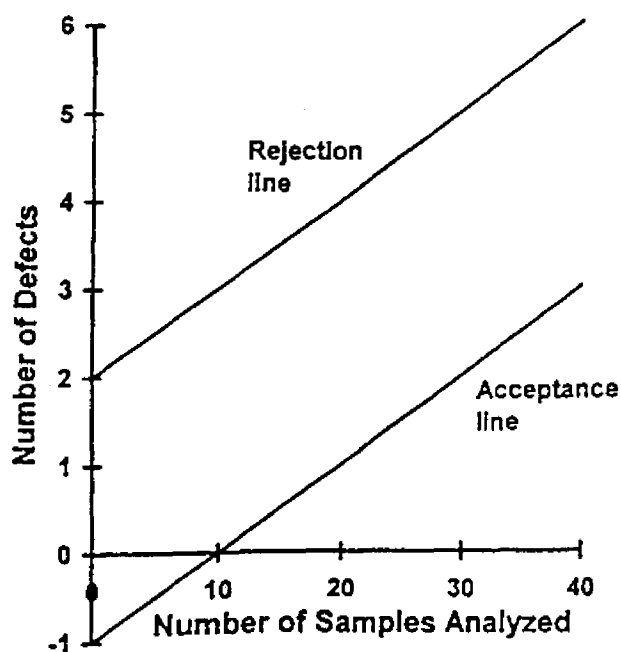
needed when determining mycotoxin levels, when sampling error is many times greater than analytical error (1). In this case, sampling and good comminution and mixing prior to particle size reduction are more important than the chemical analysis itself. Additional information on sampling for mycotoxin analysis is provided in Chapter 20 section 20.2.2.

5.2.5 Risks Associated with Sampling

There are two types of risks associated with sampling. Both should be considered when developing a sampling plan (1). The consumer risk describes the probability of accepting a poor quality population. This should happen rarely (<5% of the lots) but the actual acceptable probability of a consumer risk depends on the consequences associated with accepting an unacceptable lot. These may vary from major health hazards and subsequent fatalities to a lot being of slightly lower quality than standard lots. Obviously, the former demands a low or no probability of occurring whereas the latter would be allowed to occur more frequently. The vendor risk is the probability of rejecting an acceptable product. As with consumer risk, the consequences of an error determine the acceptable probability of the risk. An acceptable probability of vendor risk is usually 5–10%.

A sampling plan should be simple and flexible, protect both the consumer and the vendor, and provide for utilization of rejected lots (1). Further discussion of sampling plans can be found in section 5.3.3.

5.3 SAMPLING PROCEDURES

5.3.1 Introduction and Examples

The reliability of analytical data is compromised if sampling is not done properly. As shown in Table 5-1, the use of the data to be obtained will determine the sampling procedure. Details for the sampling of specific food products are described in the *Official Methods of Analysis* of AOAC International (6) and in the *Code of Federal Regulations* (CFR) (7). Two such examples for specific foods follow.

The AOAC Method 925.08 (6) describes the method for sampling flour from sacks. The number of sacks to be sampled is determined by the square root of the number of sacks in the lot. The sacks to be sampled are chosen according to their exposure. The samples that are more frequently exposed are sampled more often than samples that are exposed less. Sampling is done by drawing a core from a corner at the top of the sack diagonally to the center. The sampling instrument is a cylindrical, pointed, polished trier with a pointed end. It is 13 mm in diameter with a slit at least one third of the circumference of the trier. A second sample is

5-1 figure Acceptance/rejection chart for multiple sampling plans for use in quality assurance.

taken from the opposite corner in a similar manner. The cores are stored for analysis in a clean, dry, airtight container that has been opened near the lot to be sampled. The container should be sealed immediately after the sample is added. A separate container is used for each sack. Additional details regarding the container and the procedure also are described below.

Title 21 CFR specifies the sampling procedures required to ensure that specific foods conform to the standard of identity. In the case of canned fruits, 21 CFR 145.3 defines a sample unit as "container, a portion of the contents of the container, or a composite mixture of product from small containers that is sufficient for the testing of a single unit" (7). Furthermore, a sampling plan is specified for containers of specific net weights. The container size is determined by the size of the lot. A specific number of containers must be filled for sampling of each lot size. The lot is rejected if the number of defective units exceeds the acceptable limit. For example, out of a lot containing 48,001 to 84,000 units, each weighing 1 kg or less, 48 samples should be selected. If six or more of these units fail to conform to the attribute of interest the lot will be rejected. Based on statistical confidence intervals, this sampling plan will reject 95% of the defective lots examined, i.e., 5% consumer risk (7).

The discussion below describes general considerations to take into account when obtaining a sample for analysis.

5.3.2 Homogeneous versus Heterogeneous Populations

The ideal population would be uniform throughout and identical at all locations. Such a population would be homogeneous. Sampling from such a population is simple, as a sample can be taken from any location and the analytical data obtained will be representative of the whole. However, this occurs rarely, as even in an apparently uniform product, such as sugar syrup, suspended particles and sediments in a few places may render the population heterogeneous. In fact, most populations that are sampled are heterogeneous. Therefore, the location within a population where a sample is taken will affect the subsequent data obtained. However, sampling plans (section 5.2.2) and sample preparation (section 5.4) can make the sample representative of the population or take heterogeneity into account in some other way.

5.3.3 Manual versus Continuous Sampling

To obtain a manual sample the person taking the sample must attempt to take a "random sample" to avoid human bias in the sampling method. Thus, the sample

must be taken from a number of locations within the population to ensure it is representative of the whole population. For liquids in small containers, this can be done by shaking prior to sampling. When sampling from a large volume of liquid, such as that stored in silos, aeration ensures a homogeneous unit. Liquids may be sampled by pippetting, pumping, or dipping (Fig. 5-2). However, when sampling grain from a rail car, mixing is impossible and samples are obtained by probing from several points at random within the rail car. Such manual sampling of granular or powdered material is usually achieved with triers or probes that are inserted into the population at several locations. Errors may occur in sampling (8), as rounded particles may flow into the sampling compartments more easily than angular ones. Similarly, hygroscopic materials flow more readily into the sampling devices than do nonhygroscopic material. Horizontal core samples have been found to contain a larger proportion of smaller sized particles than vertical ones (8).

Continuous sampling is performed mechanically. Figures 5-3 and 5-4 show automatic sampling devices in production lines for liquids and solids, respectively.
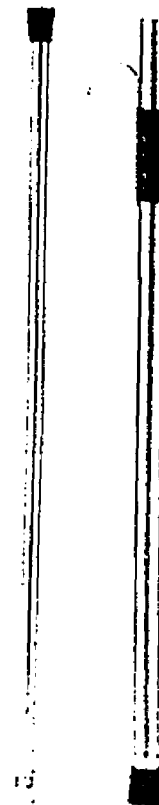
5-2
figure

A manual core sampler for fluids. The sampler takes 4 ml of fluid for each inch (2.54 cm) depth. Sampler is shown with sanitizing/carrier case (right). (Courtesy of Liquid Sampling Systems Inc., Cedar Rapids, IA.)
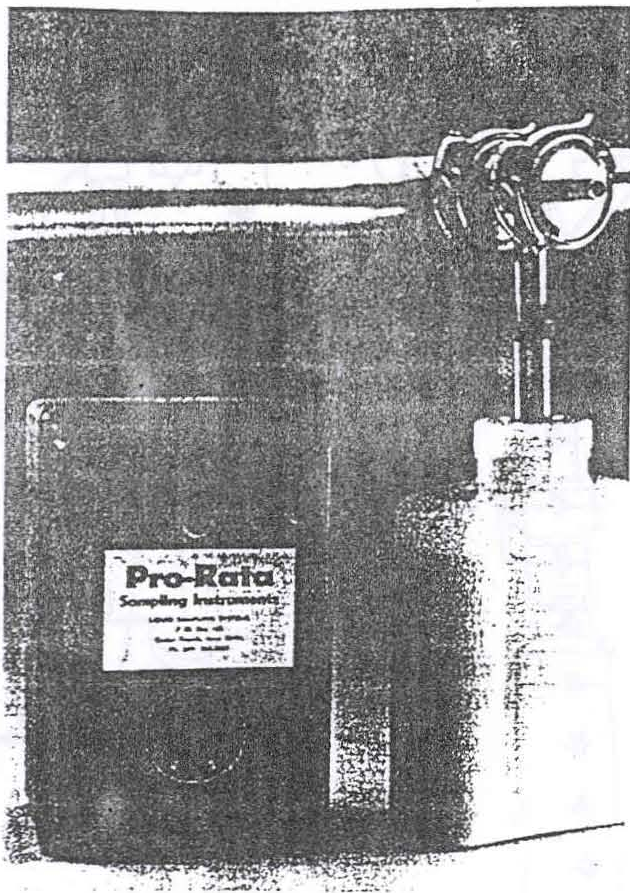
An automatic liquid sampling device that uses air under high pressure to collect multiple 1.5-mL samples. The control box (left) regulates the sampling frequency. (Courtesy of Liquid Sampling Systems Inc., Cedar Rapids, IA.)

Continuous sampling should be less prone to human bias than manual sampling.

5.3.4 Statistical Considerations

5.3.4.1 Nonprobability Sampling

Nonprobability sampling is performed when a representative sample of a population cannot be collected. In certain cases of adulteration such as rodent contamination, the objective of the sampling plan may be to highlight the adulteration rather than collect a representative sample of the population. This type of sampling can be done in many ways, but in each case the probability of including any specific portion of the population is not equal because the investigator selects the samples, without estimating sampling error.

Judgment sampling is solely at the discretion of the sampler and therefore is highly dependent on the person taking the sample. This method is used when it is the only practical way of obtaining the sample. It

may result in a better estimate of the population than random sampling if sampling is done by an experienced individual and the limitations of extrapolation from the results are understood (1). Convenience sampling is performed when ease of sampling is the key factor. The first pallet in a lot or the sample that is most accessible is selected. This also is called "chunk sampling" or "grab sampling." Although this sampling requires little effort, the sample obtained will not be representative of the population, and therefore is not recommended. Restricted sampling may be unavoidable when the entire population is not accessible. This is the case if sampling from a loaded boxcar, but the sample will not be representative of the population. Quota sampling is the division of a lot into groups representing various categories, and samples are then taken from each group. This sampling method is less expensive than random sampling but also is less reliable.

5.3.4.2 Probability Sampling

Probability sampling plans provide a statistically sound basis for obtaining representative samples with elimination of human bias (1) and therefore is the most desirable. The probability of including any item in the sample is known and sampling error can be calculated.

Simple random sampling requires that the number of units in the population be known and each unit is assigned a number. A specific quantity of random numbers between one and the total number of population units is selected. Sample size is determined by lot size and the potential impact of a consumer or vendor error. Various sampling plans are used, including random number tables and computer-generated random numbers. Units corresponding to the random numbers then are analyzed as an estimate of the population.

Systematic sampling is used when a complete list of sample units is not available, but when samples are distributed evenly over time or space, such as on a production line. The first sample is selected at random and then every nth unit after that. However, the variance is difficult to determine.

Stratified sampling involves dividing the population into overlapping subgroups so that each subgroup is as homogeneous as possible. Group means, therefore, differ from each other as much as possible. Random samples then are taken from each subgroup. The procedure provides a representative sample because no part of the population is excluded and it is less expensive than simple random sampling.

Cluster sampling entails dividing the population into subgroups, or clusters, so that the clusters' characteristics are as identical as possible, i.e., the means are as similar as possible. Any heterogeneity occurs within

Product to be sampled.

Air filter, regulator and air gauge.

Sampler is designed to fit almost any diameter vertical or horizontal pneumatic line, positive or negative pressure.

Solenoid-controlled air cylinder.

Sample tube traverses I.D. of pipe and captures sample through opening.

Sample tube grabs sample, retracts, then seals, depositing sample by gravity flow into container.

Controller is designed with any range timer and will take pre-set samples to customer requirements.

5-4 figure An automatic sampling device for powders, granules, and pellets. Sampling occurs by exerting negative or positive pressure in horizontal or vertical pneumatic conveying systems. (Courtesy of Gustafson, Inc., Dallas, TX.)

each cluster. Clusters should be small with a similar number of units in each cluster. The clusters are sampled randomly and may be either totally inspected or subsampled for analysis. This sampling method is more efficient and less expensive than simple random sampling, if populations can be divided into homogeneous groups.

Composite sampling is used to obtain samples from bagged products such as flour, seeds, and larger items in bulk. Two or more samples are combined to obtain one sample for analysis that reduces differences between samples. For example, FDA and FSIS composite 12 and at least six subsamples, respectively, for the sample to be analyzed for compliance with nutrition labeling regulations (9).

5.3.4.3 Mixed Sampling

Mixed sampling combines random and nonstatistical sampling. The population is subdivided by the investigator and items from the groups are selected randomly.

5.3.4.4 Optimum Sampling Size and Statistical Analysis

Statistical analysis, using the t-test, provides important information regarding the optimum sample size needed to obtain a reliable population estimate. This information is used to avoid wasting resources by avoiding unnecessary sampling or sampling with sample numbers too small to provide reliable data.

The sample size is dependent on how accurate the estimate needs to be, i.e., the sample size depends on the degree of accuracy required. A larger sample size is needed to obtain a population estimate that is plus or minus 5% of the true value than would be needed to obtain an estimate that is plus or minus 25%. Equation [1] shows how the optimum sample size for a certain degree of accuracy can be found using t-values.

$$t = \frac{\bar{x} - \mu}{SD/\sqrt{n}} \qquad [1]$$

where:

$\bar{x}$ = sample mean
μ = population mean
SD = standard deviation of the sample
n = sample size

To find the probability that the sample and population means are different, the calculated t-value can be compared to a t-distribution with degrees of freedom one less than the sample size. The denominator of Equation [1] (SD/ $\sqrt{n}$) is known as the standard error of the mean (SEM). The SEM is close to zero as the sample size approaches infinity. If the SEM is multiplied by the appropriate t-value, a confidence interval can be estimated. If the t-value has a 0.05 level of significance, the data have a 95% probability of being within the confidence interval, i.e., 95% confidence.

If the denominator in Equation [1] is replaced by *accuracy* × *sample mean*, the equation can be rearranged and solved for sample size, as shown below (terms are defined in Equation [1] and below):

$$\text{sample size} = (t_{\alpha,n-1})^2 \, (SD)/(\text{accuracy} \times \bar{x})^2 \qquad [2]$$

If a preliminary study is done to find the sample mean and sample variance, the equation will calculate the sample size needed for any degree of accuracy. In Equation [2], the t-value ($t_{\alpha,n-1}$) is obtained from a t-distribution with a specified significance level (e.g., α = 0.05), and degrees of freedom identical to the denominator in finding sample variance, i.e., $n - 1$. A sample mean within 10% of the population would represent an accuracy of 0.1.

The calculated sample size is most useful if the data follow a Gaussian normal distribution. Nevertheless, the central limit theorem, which states that as sample size increases the means of samples drawn from a population with any distribution will approach a normal Gaussian distribution, can be applied to populations with various statistical distributions.

5.3.5 Problems in Sampling

Analytical data never are more reliable than the sampling technique. Sampling bias, due to nonstatistically

viable convenience, may compromise reliability. Errors also may be introduced by not understanding the population distribution and subsequent selection of an inappropriate sampling plan.

Unreliable data also can be obtained by nonstatistical factors such as poor sample storage resulting in sample degradation. Samples should be stored in a container that protects the sample from moisture and other environmental factors that may affect the sample (e.g., heat, light, air). To protect against changes in moisture content, samples should be stored in an airtight container. Light-sensitive samples should be stored in containers made of opaque glass, or the container wrapped in aluminum foil. Oxygen-sensitive samples should be stored under nitrogen or an inert gas. Refrigeration or freezing may be necessary to protect chemically unstable samples. However, freezing should be avoided when storing unstable emulsions. Preservatives (e.g., mercuric chloride, potassium dichromate, and chloroform) (2) can be used to stabilize certain food substances during storage.

Mislabeling of samples causes mistaken sample identification. Samples should be clearly identified by markings on the sample container in a manner such that markings will not be removed or damaged during storage and transport. For example, plastic bags that are to be stored in ice water should be marked with water-insoluble ink.

If the sample is an official or legal sample the container must be sealed to protect against tampering and the seal mark easily identified. Official samples also must include the date of sampling with the name and signature of the sampling agent. The chain of custody of such samples must be identified clearly.

5.4 PREPARATION OF SAMPLES

5.4.1 General Size Reduction Considerations

If the particle size or mass of the sample is too large for analysis, it must be reduced in bulk or particle size (2). To obtain a smaller quantity for analysis the sample can be spread on a clean surface and divided into quarters. The two opposite quarters are combined. If the mass is still too large for analysis, the process is repeated until an appropriate amount is obtained. This method can be modified for homogeneous liquids by pouring into four containers, and can be automated (Fig. 5-5). The samples are thus homogenized to ensure negligible differences between each portion (1).

AOAC International (6) provides details on the preparation of specific food samples for analysis, which depends on the nature of the food and the analysis to be performed. For example, in the case of meat and meat products (6), it is specified in Method 983.18 that small samples should be avoided, as this results in

Stream of material

Rotating
tube

Motor

Laboratory   Remainder
sample   of material
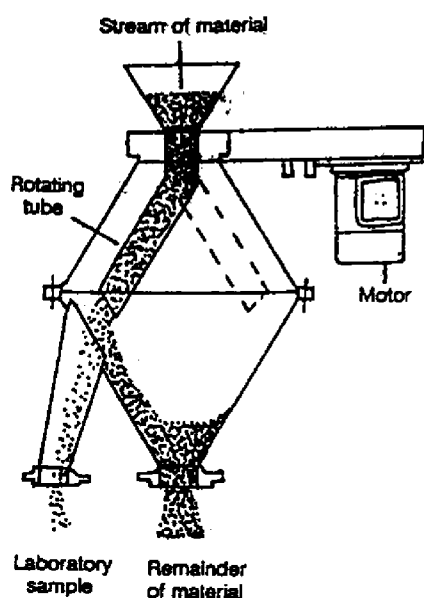
5-5
figure

A rotating tube divider for reducing a large sample (ca. 880 kg) of dry, free flowing material to a laboratory size sample (ca. 0.2 kg). (Courtesy of Glenn Mills, Inc., Clifton, NJ.)

significant moisture loss during preparation and subsequent handling. Ground meat samples should be stored in glass or similar containers, with air- and watertight lids. Fresh, dried, cured, and smoked meats are to be bone free and passed three times through a food chopper with plate openings no more than 3 mm wide. The sample then should be mixed thoroughly and analyzed immediately. If immediate analysis is not possible, samples should be chilled or dried for short-term and long-term storage, respectively.

A further example of size reduction is the preparation of solid sugar products for analysis as described in AOAC Method 920.175 (6). The method prescribes that the sugar should be ground, if necessary, and mixed to uniformity. Raw sugars should be mixed thoroughly and rapidly with a spatula. Lumps are to be broken by a mortar and pestle or by crushing with a glass or iron rolling pin on a glass plate.

5.4.2 Grinding

Various mills are available for reducing particle size for sample homogenization (9). To homogenize moist samples, bowl cutters, meat mincers, tissue grinders, mortars and pestles, or blenders are used, while mortars and pestles and mills are best for dry samples.

Mills differ according to their mode of action, being classified as a burr, hammer, impeller, cyclone, impact, centrifugal, or roller mill (10). Methods for grinding dry materials range from a simple pestle and mortar to power driven hammer mills. Hammer mills

wear well, and reliably and effectively grind cereals and dry foods, while small samples can be finely ground by ball mills. A ball mill grinds by rotating the sample in a container that is half filled with ceramic balls. This impact grinding can take hours or days to complete. A chilled ball mill can be used to grind frozen foods without predrying and also reduces the likelihood of undesirable heat-initiated chemical reactions occurring during milling (10). Alternatively, dry materials can be ground using an ultracentrifugal mill by beating, impact, and shearing. The food is fed from an inlet to a grinding chamber and is reduced in size by rotors. When the desired particle size is obtained, the particles are delivered by centrifugal force into a collection pan (10). Large quantities can be ground continuously with a cyclone mill.

Particle size is controlled in certain mills by adjusting the distance between burrs or blades or by screen mesh sizes (i.e., the number of openings per linear inch of mesh). The final particles of dried foods should be 20 mesh for moisture, total protein, or mineral determinations. Particles of 40 mesh size are used for extraction assays such as lipid and carbohydrate estimation.

Some foods are more easily ground after drying in a desiccator or vacuum oven. Grinding wet samples may cause significant losses of moisture and chemical changes. In contrast, grinding frozen samples reduces undesirable changes.

The grinding process should not heat the sample, and therefore the grinder should not be overloaded to control the heat produced through friction. Contact of food with bare metal surfaces should be avoided if trace metal analysis is to be performed.

To break up moist tissues, a number of slicing devices are available; bowl cutters can be used for fleshy tubers and leafy vegetables while meat mincers may be better suited for fruit, root, and meat (10). Addition of sand as an abrasive can provide further subdivision of moist foods. Waring blenders are effective in grinding soft and flexible foods and suspensions. Rotating knives (25,000 rpm) will disintegrate a sample in suspension. In colloidal mills, a dilute suspension is flowed under pressure through a gap between slightly serrated or smooth surfaced blades until they are disintegrated by shear. Sonic and supersonic vibrations disperse foods in suspension and in aqueous and pressurized gas solution. The Mickle disintegrator sonically shakes suspensions with glass particles and the sample is homogenized and centrifuged at the same time (10). Alternatively, a low shear continuous tissue homogenizer is fast and handles large volumes of sample.

5.4.3 Enzymatic Inactivation

Food materials often contain enzymes that may degrade the food components being analyzed. Enzyme

activity therefore must be eliminated or controlled using methods that depend on the nature of the food. Heat denaturation for enzyme inactivation and freezer storage (–20 to –30°C) for limiting enzyme activity are common methods. However, some enzymes are more effectively controlled by changing the pH, or by salting out (10). Oxidative enzymes may be controlled by adding reducing agents.

5.4.4 Lipid Oxidation Protection

Lipids present particular problems in sample preparation. High fat foods are difficult to grind and may need to be ground while frozen. Unsaturated lipids are sensitive to oxidative degradation and should be protected by storing under nitrogen or vacuum. Antioxidants may stabilize lipids and may be used if they do not interfere with the analysis. Light-initiated photooxidation of unsaturated lipids can be avoided by controlling storage conditions. In practice, lipids are more stable when frozen in intact tissues rather than as extracts (10). Therefore, ideally, unsaturated lipids should be extracted just prior to analysis. Low-temperature storage is generally recommended to protect most foods.

5.4.5 Microbial Growth and Contamination

Microorganisms are present in almost all foods and can alter the sample composition. Likewise, microorganisms are present on all but sterilized surfaces, so sample cross-contamination can occur if samples are not handled carefully. The former is always a problem and the latter is particularly important in samples for microbiological examination. Freezing, drying, and chemical preservatives are effective controls and often a combination of these is used. The preservation methods used are determined by the probability of contamination, the storage conditions, storage time, and the analysis to be performed (10).

5.5 SUMMARY

Food quality is monitored at various processing stages but 100% inspection is rarely possible, or even desirable. To ensure a representative sample of the population is obtained for analysis, sampling and sample reduction methods must be developed and implemented. The selection of the sampling procedure is determined by the purpose of the inspection, the food product, the test method, and the characteristics of the population. Increasing the sample size will generally increase the reliability of the analytical results and using t-test techniques will optimize the sample size necessary to obtain reliable data. Multiple sampling techniques also can be used to minimize the number of

samples to be analyzed. Sampling is a vital process, as it is often the most variable step in the entire analytical procedure.

Sampling may be for attributes or variables. Attributes are monitored for their presence or absence, whereas variables are quantified on a continuous scale. Sampling plans are developed for either attributes or variables and may be single, double, or multiple. Multiple sampling plans reduce costs by rejecting low-quality lots or accepting high-quality lots quickly, while intermediate quality lots require further sampling. There is no sampling plan that is risk free. The consumer risk is the probability of accepting a poor quality product, while the vendor risk is the probability of rejecting an acceptable product. An acceptable probability of risk depends on the seriousness of a negative consequence.

Sampling plans are determined by whether the population is homogeneous or heterogeneous. Although sampling from a homogeneous population is simple, it rarely is found in practical industrial situations. Sampling from heterogeneous populations is most common and suitable sampling plans must be used to obtain a representative sample. Sampling methods may be manual or continuous. Ideally, the sampling method should be statistically sound. However, nonprobability sampling is sometimes unavoidable, even though there is not an equal probability that each member of the population will be selected due to the bias of the person sampling. Probability sampling is preferred because it ensures random sampling and is a statistically sound method that allows calculation of sampling error and the probability of any item of the population being included in the sample.

Each sample must be clearly marked for identification and preserved during storage until completion of the analysis. Official and legal samples must be sealed and a chain of custody maintained and identified. Often, only a portion of the sample is used for analysis and sample size reduction must ensure that the portion analyzed is representative of both the sample and population. Sample preparation and storage should account for factors that may cause sample changes. Samples can be preserved by limiting enzyme activity, preventing lipid oxidation, and inhibiting microbial growth/contamination.

5.6 STUDY QUESTIONS

1. As part of your job as supervisor in a quality assurance laboratory, you need to give a new employee instruction regarding choosing a sampling plan. Which general factors would you discuss with the new employee? Distinguish between sampling for attributes versus sampling for variables. Differentiate the three basic sampling plans and the risks associated with selecting a plan.
2. Your supervisor wants you to develop and implement a

multiple sampling plan. What would you take into account to define the acceptance and rejection lines? Why?

3. Distinguish nonprobability sampling from probability sampling. Which is preferable and why?

4. (a) Identify a piece of equipment that would be useful in *collecting* a representative sample for analysis. Describe precautions to be taken to ensure a representative sample is taken and a suitable food product that could be sampled with this device. (b) Identify a piece of equipment that would be useful for *preparing* a sample for analysis. What precautions should be taken to ensure the sample composition is not changed during preparation?

5. For each of the problems identified below that can be associated with collection and preparation of samples, state one solution for how the problem can be overcome:
   a. Sample bias
   b. Change in composition during storage of sample prior to analysis
   c. Metal contamination in grinding
   d. Microbial growth during storage of product prior to analysis

6. The instructions you are following for cereal protein analysis specify grinding a cereal sample to 10 mesh before you remove protein by a series of solvent extractions.
   a. What does 10 mesh mean?
   b. Would you question the use of a 10 mesh screen for this analysis? Provide reasons for your answer.

7. You are to collect and prepare a sample of cereal produced by your company for the analyses required to create a standard nutritional label. Your product is considered "low fat" and "high fiber" (see regulations for nutrient claims, and FDA compliance procedures in Chapter 3). What kind of sampling plan will you use? Will you do attribute or variable sampling? What are the risks associated with sampling in your specific case? Would you use probability or nonprobability sampling, and which specific type would you choose? What specific problems would you anticipate in sample collection and in preparation of the sample? How would you avoid or minimize each of these problems?

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ACKNOWLEDGMENT

chapter

Computerization and Robotics

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6.1 COMPUTERS FOR DATA ACQUISITION

The promise of computers, robotics, and automation is to relieve the scientist from tedious and undesirable tasks. These might include the three Ds—dirty, dull, and dangerous—to say nothing of demeaning and debilitating. Apart from the need for computers to do repetitive tasks quickly and efficiently, many tasks can be accomplished with great speed, accuracy, and precision. At present the food scientist can be almost overwhelmed in terms of the choices available for laboratory needs. There is a modern bromide about computers, termed the "18-month rule." This refers to the past decade's observation that within 18 months, virtually all computers and their software will become obsolete, or at least no longer state-of-the-art. The authors of this chapter are painfully aware of this phenomenon and thus will strive to stay with broad-based concepts that encompass a few turns of the 18-month rule.

6.1.1 Historical Overview

The advent of modern computers in the food scientist's laboratory has been made possible by rapidly decreasing costs of computer hardware packaged in smaller physical size, but with ever increasing computational power. Vendors of analytical equipment such as spectrophotometers, gas chromatographs (GCs), or robotic autosamplers, among many others, have incorporated microprocessors in most modern instrumentation and robotics systems. Vendors usually supply a system as a ready-to-use or turnkey product. Often vendors make available optional interfaces to attach to computers for customized needs in the laboratory. An example of such a system might be a GC system in which a microprocessor monitors and controls operations of the GC and then sends a filtered and conditioned signal to a recording device. Typically, this is in the form of a recording integrator, in which the chromatographic peaks are integrated by another dedicated microprocessor and a tracing of the chromatogram is provided along with a printed copy of integrated peak areas. The user often has very little optional, direct control of such a system unless further connection and interfacing with a separate computer are possible and desirable.

A further step upward in data acquisition is larger computer systems that provide a chemical workstation. These can provide computer control and acquisition of data from multiple instruments, often simultaneously. However the turnkey systems can be very costly and more than is needed for a specific assay.

If the user finds that the turnkey product or chemical workstation choices are unacceptable for specific needs, then it may be necessary to design a dedicated computer for customized use in the laboratory. In contrast to the dedicated turnkey environment described previously, the next step in automation is to make connection directly to the experiment with a laboratory computer. This often is desirable for long-term storage of data and further treatment of experimental data. An example would be to interface a computer to a GC and integrator. The GC and integrator each contain their own microprocessors. However, the computer controls each of these dedicated instruments and captures the GC signal along with the results of the integrator's calculations of retention times and peak areas. An example is shown in Fig. 6-1 of experimental results from such an experimental configuration that was used to capture all relevant data for GC analyses of tomato headspace volatiles. Advantages of such a system include a lower cost of operation, along with permanent long-term and easily retrievable data for both the signal and the integrator report. In addition, the signal can be regraphed with differing amplitudes in a customized form by a graphics program such as Igor[TM], from which Fig. 6-1 was taken.

6.1.2 Hardware Requirements

6.1.2.1 Computers

Choices of computers for the laboratory can be overwhelming. Computers for laboratories first became available in the 1970s largely through minicomputer products from Digital Equipment Corporation (DEC). In the mid-1980s IBM PC microcomputers became widely available, especially with the introduction of low-cost clones that now dominate the marketplace in the 1990s. Apple Macintosh[®] computers have value for educational institutions because of flexibility with some applications and ease of use. Choice of computers should be based on the food scientist's experimental needs, such as the type of direct connections or interfacing necessary to monitor and control instruments or experiments in the laboratory.

6.1.2.2 Transducers

If an analytical apparatus is not directly computer compatible, it is necessary to use the correct transducer to produce an electrical signal for each physical parameter to be measured. For example, to measure temperature, thermocouples are often employed. The resulting thermocouple electrical signals need to be amplified, filtered, and conditioned to provide a reproducible and stable electrical signal. Fortunately, to find an appropriate transducer the food scientist can consult a number of vendors who merely need a description of the experimental requirements.

exchange protocol termed either IEEE-488 or GPIB requires a separate interface card. This latter protocol has been adapted for thousands of instruments, and many computer programs have been written to accommodate this standard protocol, independent of specific computers or instruments.

Often, the microcomputers themselves are interfaced together and can thus acquire and transmit data simultaneously. The use of local area networks (LANs) is becoming common. The use of LANs within laboratory information management systems (LIMS) is discussed in section 6.3.3.1.

6.1.3 Software Requirements

6.1.3.1 Real-Time and Post-Run

If computers are used to analyze data after the physical or chemical measurements are completed, the analysis is said to be post-run. This is a common application of computers; the data are either typed by hand, input through a digitizing tablet, or perhaps entered in graphic form from a commercial scanner/digitizer. No matter what the form of data entry, the scientist must be vigilant to monitor errors during the process. (Estimates of typed error rates are commonly given as from 3% to 15%.)

A compelling reason for directly interfacing a computer to an instrument or experimental apparatus is to reduce such data recording errors. Ideally, a computer is able to input the data immediately and at the same time as it is acquired experimentally; this is termed real-time data acquisition. Since the data ultimately must be transmitted in digital form as bytes or words of information, the rate at which computers can do real-time acquisition may be limiting to the experiment; however, it is not uncommon to acquire data points at rates of 500 kHz or more in instruments such as those used for resonance imaging (see Chapter 30).

6.1.3.2 Interface Hardware and Drivers

The data can arrive at the computer interface in different formats and need to be converted into an appropriate form for further use and storage. Incoming data may be binary, binary coded decimal (BCD), American Standard for Information Interchange (ASCII), or Gray code, among others. Interface cards can do required conversions directly or otherwise transmit the data for further handling and storage by software in the computer.

Drivers are specialized software programs that provide the link between the hardware interface and more advanced software or programs that handle, manipulate, display, and store the data. Driver programs are designed to provide the most efficient data

exchange possible and at the highest possible speed. They are the first link between the hardware and computer software. Drivers are written in the most concise computer code possible (assembly or machine language) and are not usually changed or reprogrammed by users of the driver who wish only to link their more sophisticated programs to the driver.

Many software vendors can provide the laboratory scientist with the software and drivers necessary for their experiments. A cursory search of the Internet will return virtually thousands of vendors. For customized needs, National Instruments Corp. provides state-of-the-art programs, LabVIEW™ and LabWINDOWS™, for most lab computer platforms. These products have used a development environment in which the user designs a customized interface to the experiment using graphical icons that simply describe the needs of the interface. This builds a virtual instrument for testing and refinement. Then an interface/driver icon is simply inserted into the program that attaches the drivers to interface cards and instrument. The difficult computer programming is transparent. An example of a LabVIEW program application is shown in Fig. 6-2; it shows the computer screen display for the interface and controller of an oxygen electrode apparatus used to measure oxygen uptake during accelerated lipid oxidation experiments. (This was such a specialized apparatus and experimental protocol that no vendor-supplied software existed). The user simply clicks a mouse pointer on the appropriate controls to change settings, and instantly the entire experiment is under computer control. Graphical results and calculations are displayed in real-time and data are stored simultaneously for archive purposes.

6.1.3.3 Integration into Software Programs

Directly incoming (real-time) data or stored data need to be treated with software programs. The use of once-formidable programming languages such as Basic, Fortran, Pascal, C, or C++ is no longer the rule for analyzing data with software. Many specialized programs for handling data exist that do not require extensive programming skills. The concept and development of user-friendly programs have taken the laboratory computer to new levels of ease of use.

6.1.4 Ergonomics and Economics

The modern computer system can be purchased as part of packaged commercial instruments and apparatus. When commercial products do not meet the exact needs of the food scientist, easily adaptable computer systems and programs are available that need not be intimidating to the nonprogrammer of computers. The products discussed previously, along with a myriad
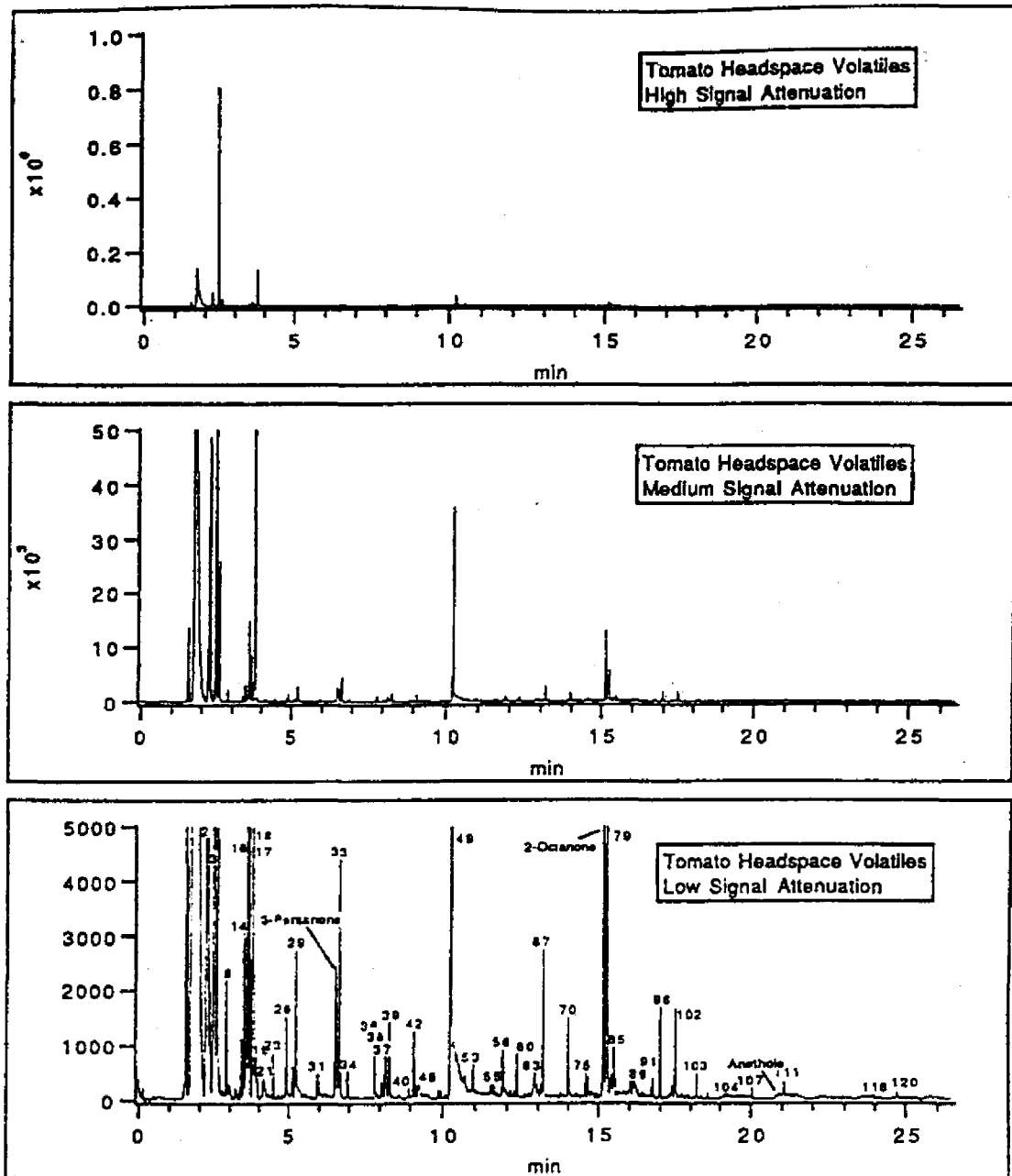
Figure 6-1 Layout of three chromatograms produced by the graphics program Igor™ that displays three different signal amplification levels or degrees of attenuation.

If the transducer produces an analog electrical signal, this signal must be converted to a digital form to be appropriate input for modern digital computers. Modern analog-to-digital converters (ADCs) are available in convenient form as boards or cards that merely need be inserted into the backplane or bus slots on most computers. Often, a single multipurpose interface card will serve all the experimental needs of an analytical laboratory; however, appropriate software must be available to use it effectively.

6.1.2.3 Interfaces with the Computer

If an instrument or experiment's transducers already provide a digitized signal, interfacing can be accomplished directly through a serial port provided on most computers. The exact protocol for sending and receiving this digital information needs to be defined, and there exist many protocols for the lab computer. The protocols defined as EIA RS-232c and RS-423 are common for IBM and Macintosh computers. Another data

Oxygen Electrode Read Panel

Instrument Settings:

Power   Save File

Stop [   ] Start   No [   ] Yes

Sampling Frequency   Sampling Frequency
for O2 Absorption (Hz)   for Base Line (Hz)

[1   ]   [120   ]

Absolute Zero   Lipid Zero
                         Loop Drive

[320   ]   [325   ]

Points For B-line   1/2 Time of
Determination   Control

[1200   ]   [75   ]

Instrument Output:

1/2 Time Of Sample   Antioxidant Effect

[105.00   ]   [0.40   ]

0. —
1. —   O2 Saturation Level
2. —   [2019.26   ]
3. —
4. —   Standard Deviation
5. —   [2.23   ]

Sample Oxygen Consumption Curve

6-2 figure   A grayscale screen capture of a full-color LabVIEW™ interface to an oxygen electrode experimental apparatus. The experimental parameters are set directly from this screen display. The results and calculations are displayed, as shown, in real-time. Results of up to six experiments are displayed in separate colors on the computer display.

others, offer easy to use products with graphical and visually aesthetic programs. Ultimately, the promise of error-free data acquisition and the speed of computers make the use of computers in the laboratory very cost effective and can free the food analyst for other tasks. More importantly, it is becoming more common that analytical laboratories are required to be certified as compliant with government and agency standards for their analyses. Computerized and automated analytical information that provides error-free data with certified audit-trail security will become commonplace.

6.2 COMPUTERS FOR DATA ANALYSIS AND DISPLAY

The power of computers has become available in surprisingly small physical packages that engender tremendous calculation power. Important uses of laboratory computers include computations, graphics, and database management.

For calculations and computational needs, the use of spreadsheet programs can serve most needs of the

food scientist. Popular spreadsheet programs such as Microsoft Excel contain virtually all the scientific functions needed for most physical and chemical analyses. The food scientist needs very little computer programming expertise to create custom applications from spreadsheet programs that handle all needed calculations and formatting for reports.

If a picture is worth a thousand words, the use of graphics is one of the most valued reasons for the food scientist to use computers in the laboratory. From the graphing of experimental results to programs that draw chemical structures for reports, specialized programs abound. Most spreadsheets also have limited graphing capabilities, but they may not be totally adequate. For example, results of an enzyme assay could easily be calculated and displayed from a spreadsheet program. However, as an alternate method, a specific product written for enzyme experiments, such as EnzymeKinetics™ for the Macintosh, instantaneously can do required calculations and print graphs such as shown in Fig. 6-3.

Many more sophisticated graphics programs, beyond the capabilities of spreadsheets and suitable

Example of Lineweaver-Burk graph
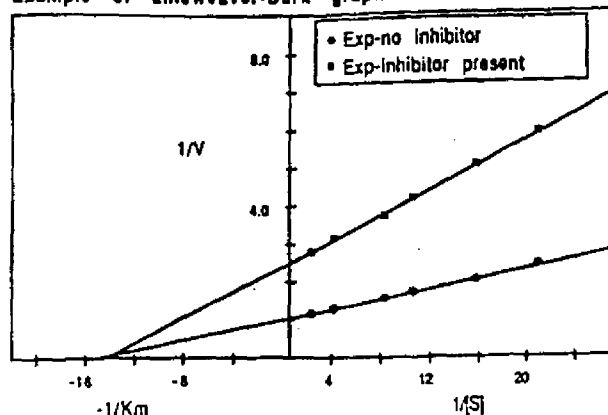
6-3 figure Graphical presentation of calculations from the EnzymeKinetics™ program.

for scientists, are available. One such example (and at the moment available only on Macintosh) is Igor™, which carries graphics and other functions far beyond the most sophisticated spreadsheet programs. Calculations and graphics for extremely large and extensive data sets are especially easily handled with the Igor™ program in forms designed for scientists, as opposed to the business-oriented applications of many graphics programs. Fourier transformations, curve fitting, data smoothing, and plotting of multiple data sets are all quickly accomplished by clicking on appropriate pull-down menus. It also can be directly interfaced to instruments and other programs and databases. The GC graph or chromatogram shown in Fig. 6-1 is an example of a plot of over 32,000 data points.

Database management can be accomplished efficiently with many programs for the lab computer. Because of the needs of the business environment, many popular database programs are available with varying capabilities from flat to relational databases. Database management programs also are bundled into laboratory information management systems (LIMS), as described in section 6.3.3.1. Statistical calculations are accomplished easily in spreadsheets for all but the most sophisticated applications. For the more demanding statistical needs, commercial programs bring the traditional mainframe computer power of programs such as SAS, MINITAB, and MATLAB to the laboratory microcomputer.

6.3 COMPUTERS FOR AUTOMATION AND ROBOTICS

An ever-increasing analytical sample load and the need for improved quality of information will require solutions that are highly productive and cost effective. The food laboratory of the future will be a highly inte-

grated, information-intensive domain that will combine sophisticated analytical techniques with automated robotic procedures.

Advancements of computer technology in both hardware and software have made a considerable impact in the way laboratory procedures are performed today. The usefulness of the computer in the laboratory has been extended not only to include the acquisition and processing of analytical data, but in many cases, it has helped to replace the physical work of sample preparation as well.

6.3.1 Laboratory Automation

The analytical food scientist of today is faced with ever-increasing quantities of samples, tests, and test replicates required to complete a desired experiment. Specialized tools are needed to overcome the mundane and repetitive tasks that often confront the food scientist. To help meet these needs, analytical instrument makers were challenged by the task of converting simple analytical tools into sophisticated computer-driven analytical data stations.

6.3.1.1 Analytical Methods

Automation opportunities were created by industries in which the demand for high sample throughput provided the economic justification to purchase or build special instrumentation that met the demand. The areas of industry that had the most to gain by laboratory automation included industrial quality control.

Steel mills, for instance, required fast and accurate analysis of the molten steel before it was poured into ingots. Without automated multielement analyzers, a proper assay of the metal would take several hours instead of seconds to perform.

Medical and pharmaceutical testing laboratories quickly caught on to laboratory automation. Obtaining fast and accurate results from a hospital's medical laboratory are important, and much of today's medical laboratory instrumentation is geared for multiple testing of biologicals with emphasis on data system integration.

The analytical methods that were commonly automated included spectrometry [ultraviolet-visible, atomic absorption spectroscopy (AAS), inductively coupled plasma-atomic emission spectroscopy (ICP-AES) (see Chapters 26 and 28)] and chromatography (see Chapters 31–33), in which much of the automation simply meant introducing a large number of prepared samples to the instrument.

6.3.1.2 Instrument Interfaces

The introduction of the computer to analytical instrumentation has changed forever the way the analytical

chemist approaches the bench. In many cases, much of the time-consuming sample preparation and measurement is performed by a machine under the control of a computer.

The most basic instrument interfaces are sample delivery systems or autosamplers that eliminate the repetitive work required to introduce a large number of samples. Usually there is some preparation work to be done before analysis. After groups of samples are arranged in a logical order, the analysis of each sample can be allowed to continue without the analyst's intervention. Complete analytical computer systems have been linked to these delivery systems so that once the samples are added to a sample tray or carousel, the system automatically performs the analysis. Options for data reduction and report generation also can be included.

An example of an automated analytical system is shown in Fig. 6-4. The analytical instrument is a gas chromatograph equipped with a multiple sample carousel. The instrument can select each sample from the carousel, inject it into the column, and run the chromatogram for a specified time. The computer interface is installed on the instrument to interpret chromatographic peak information (data reduction) and enter this information in a database for reporting purposes.

Automation systems such as the one in Fig. 6-4 are available for many analytical techniques including high performance liquid chromatography (HPLC), AAS, and ICP-AES. Somewhat more advanced instrument interfaces may include an additional step prior to analysis. One such device, known as an autoanalyzer, utilizes spectrophotometric analysis of liquid samples that are pumped through a special flow cell. Before the liquid reaches the cell, the flowing liquid sample can be subjected to various preparation steps, including the introduction of specific reagents within the stream.

6.3.2 Laboratory Robotics

Although the automation tools available to the analytical chemist have reduced some of the manual labor, many additional methods, especially in the food industry, require far more manual preparation steps that are not performed by any of today's analytical instrumentation. As advances in technologies continue into the twenty-first century, there is a clear need to automate entire procedures and give the scientist more time to continue the thinking part of the analytical procedure.

There are three steps in an analytical method that can be automated (see Fig. 6-5). The analytical chemist follows these steps in this order: (1) prepare the sample, (2) analyze the sample, and (3) reduce and report the data. In the early days of laboratory automation, the third step was automated first. In time, the second step involving automated analytical equipment became

Computer Interface
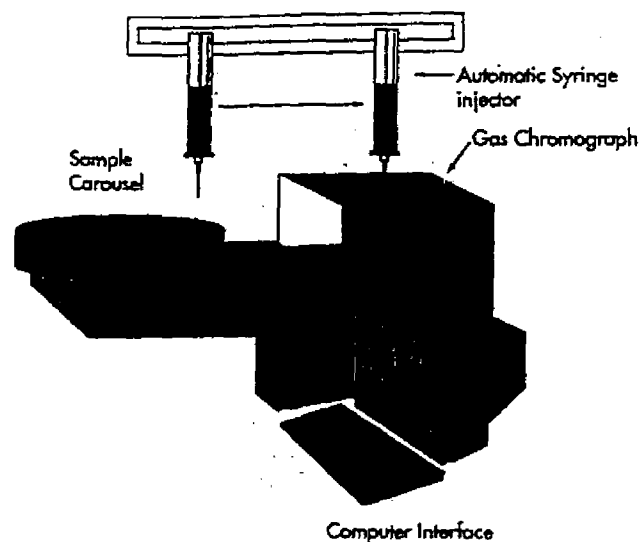
6-4 Multiple sample gas chromatographic analyzer.

more sophisticated, and direct linkage between analysis and data reduction in one instrument became a reality. Finally, when analysis and data reduction are completely automated, the next logical step will be to automate sample preparation. As more sample preparation and analysis techniques become fully automated, more emphasis can be focused on the decision-making process.

6.3.2.1 Early Development

A laboratory robot is defined as a programmable manipulator that is an extension of the capabilities present in laboratory autosamplers and autoanalyzers. It adds versatility because it can be programmed to perform a number of preparation steps over a range of samples and methods. A more generic class of autosamplers was introduced to perform a wider range of methods, but until that time, none of these systems could have been called a robot.

One of the first commercially successful laboratory robotic systems was introduced in the mid-1970s. The robot was specifically designed for the laboratory, utilized a modular system for building laboratory methods, and used a robot program language designed with the chemist in mind. This modular approach to laboratory robotics was important to the success of these early systems because it simplified the task of operating a robot in the laboratory. Many scientists found that they could easily understand the complexities of automation when each function was broken down into separate procedures or modules that could be linked together to form an entire automation sequence. Other robotic systems followed, and many laboratories designed their

SAMPLE PREPARATION

ANALYSIS

DATA REDUCTION AND DOCUMENTATION
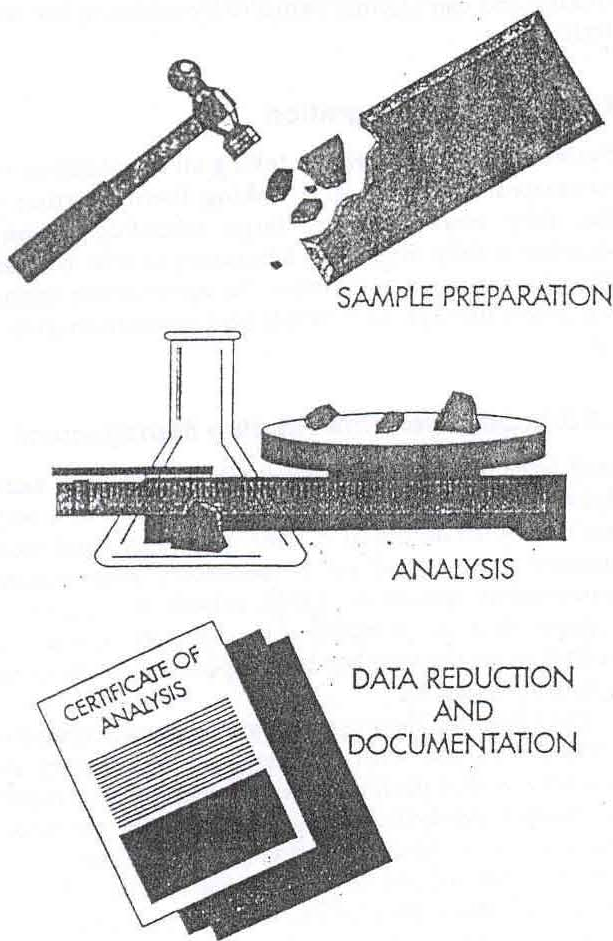
6-5 figure

Steps in analytical method that can be automated.

own specific automation requirements around one or more of the commercially available robot arms.

6.3.2.2 Mechanical Considerations

An increased use of laboratory robotics over the past 20 years is unquestionably the result of the advancement of the integrated circuit (IC) or microchip. The major IC advances have flourished not only in the function of the central processing units, or computers on a chip, but also for other IC devices. Many kinds of sophisticated integrated circuits have been introduced to aid in getting the computer to do physical labor.

Unfortunately, the advancement of mechanical robotic technology has not kept pace with that of the computer. Many of the robotic arm mechanisms on the market still utilize the electromechanical technology that was common over 50 years ago. Smaller and more powerful drive mechanisms need to be developed for robot arms. Also, robots need to be more aware, as it

were, of their surroundings; robot sensors have to be more advanced in order for the robot to move about in the real world.

6.3.2.2.1 Robotic Drive Mechanisms The drive method behind the laboratory robot arm is an important factor in choosing a specific robot application. The four main drive methods currently available are hydraulic, pneumatic, DC electric motor, and electric stepping motor drives.

Hydraulic and pneumatic drives are seldom used in analytical laboratories and are used primarily in large industrial applications in which heavy lifting or explosive atmospheres are factors. The two remaining methods are the drives that make up virtually all of the laboratory robots in use today and are based on electric motor drive technology.

DC electric motors are particularly well known motor drive mechanisms. They are feedback loop controlled motors that have exceptionally high torque-to-volume ratios that make them ideal for use in robot wrist and finger mechanisms. Many small drive systems of this type are used to drive the pen in the familiar laboratory strip chart recorders; larger versions now are being used to move robot arms for automated chemical analysis.

Electric stepper motors have several advantages over DC motors; they are inexpensive and do not require costly feedback loop control hardware. Their low torque-to-volume ratio makes them less desirable for small- and medium-size robot applications, but these motors are ideal for driving positioning tables and gantries as part of the automation system.

6.3.3.2.2 Sensors A robot that does not know where it is going in a laboratory will have trouble performing an analysis. Sensors of various types must tell the robot system the obvious: Is the hand open or closed? Is the arm near the balance? Is the test tube rack full or empty?

Position sensors are the most basic of the robotic sensors. All robotic systems have some means of determining where one part of the robot is relative to the other robot parts. This position information, although helpful, still does not give an adequate picture of the outside world; additional sensor information is necessary to help guide the robot through its motions.

True robotic vision is not yet perfected, so the robot must "see" using other means. Force detection and proximity switch activation, for example, can give a sense of touch to the robot. Sensors determine the position of the robot and the position of the items around it, but it also is necessary to incorporate sensors that will recognize failed attempts during certain critical activities. Common questions such as, Did the robot add enough solvent? or Is the tube placed properly in the centrifuge? can be answered with the use of sensors.

6.3.2.3 Laboratory Devices

The laboratory robot is not a substitute for a laboratory scientist. It is merely a tool to help the scientist be more productive. Like any sophisticated tool, a robot must have a built-in relationship with its surroundings. One cannot simply install a robot arm onto a laboratory bench and expect it to perform its job using laboratory equipment and methods that were designed for humans to use. This laboratory equipment or laboratory device must be manufactured specifically for automation purposes.

An example of a laboratory device is an analytical balance with automated doors that open when the robot is ready to weigh a sample. Laboratory devices also include laboratory glassware and disposables that the robots must use. It is important that the glassware and disposables are absolutely free of defects and are made to exacting tolerances. The robot may not be able to detect a cracked tube or a poorly molded pipette tip, so it is important that these items are properly made and easy for the robot to use.

6.3.2.4 Applications

Robots have successfully found their way into the food analysis laboratory. The applications are few, but much attention has been placed on a number of methods that have been duplicated in several food laboratories, including vitamin, sugars, fiber, and fat analyses. In addition to these applications, many preanalysis sample preparation robots routinely prepare samples for analysis by GC, HPLC, and ICP-AES.

The early laboratory robot systems were designed to interact with a number of instruments so that it would be possible to use the same robot to perform a number of different laboratory tasks. A large robot bench populated with laboratory devices (see Fig. 6-6) would be able to prepare samples for several different HPLC methods by selecting the proper software program. Other systems, such as the ICP preparation robot (Fig. 6-7), were used to prepare samples for acid digestion and wash glassware while the samples were digested.

In recent years, many of the large laboratory robot systems that featured articulated robotic arms such as the ones shown in Fig. 6-6 and 6-7 have been replaced by smaller automated workstations. These workstations have been gaining in popularity due to their smaller size and proportionately smaller price tags.

Figure 6-8 is an example of an automated workstation that simply weighs small sample containers. The sample position and weight information are stored on a computer. More complicated workstations such as the chromatographic workstation in Fig. 6-9 can combine a number of steps such as reagent addition and

mixing and can identify samples by utilizing bar code technology.

6.3.3 Systems Integration

Systems integration means taking all the pieces of the automated laboratory and linking them together so that they work like one large smoothly running machine. A fully integrated laboratory is able to access incoming samples, determine the appropriate action, and follow through with timely and accurate analytical data.

6.3.3.1 Laboratory Information Management

Food laboratories must keep track of incoming samples and be able to store the analytical data in a way that is easy to access at a later date. This analytical information is stored on a laboratory information management system, or LIMS, which is a database program that is available on any size computer depending on the number of samples and number of users of the system.

The LIMS can be accessed by the user community or those involved with the analytical laboratory in some way, so that the exchange of information is rapid and efficient. Analysts can check the LIMS to see what kind of work is required on the samples. Clients can check the LIMS to review the analytical results. Administrators can check the LIMS to see how many samples pass through the laboratory.

From a systems integration standpoint, the LIMS computer and the automated system computers can be linked together to form an integrated analytical system. Many laboratories have extended their local area networks (LANs) even further by connecting them with other local networks within the company to form an intranet network. Using this intranet, scientists throughout the company can access analytical data via a common web browser without having to run a separate database program. The result of this integration not only refines data handling but also extends the capabilities of laboratory information management by making it easy to access by larger groups of people.

6.3.3.2 Artificial Intelligence and Expert Systems

The vast power available from computer technology has helped bring forth a new age in programming capability. One area that has received a great deal of interest recently is AI, or artificial intelligence, and its more application-oriented sibling, expert systems.

Expert systems involve gaining experience in a specific area of knowledge. From this base of knowledge can be formed a method for properly coordinat-
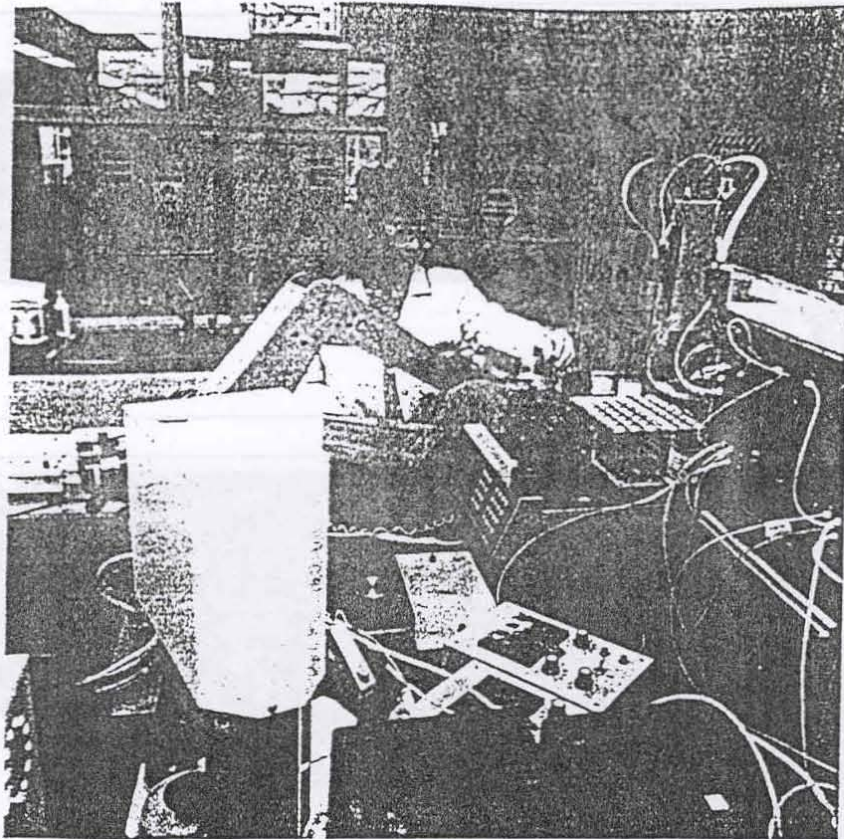
6-6 *figure* A laboratory robot system is trained to locate one of the laboratory devices on the bench.
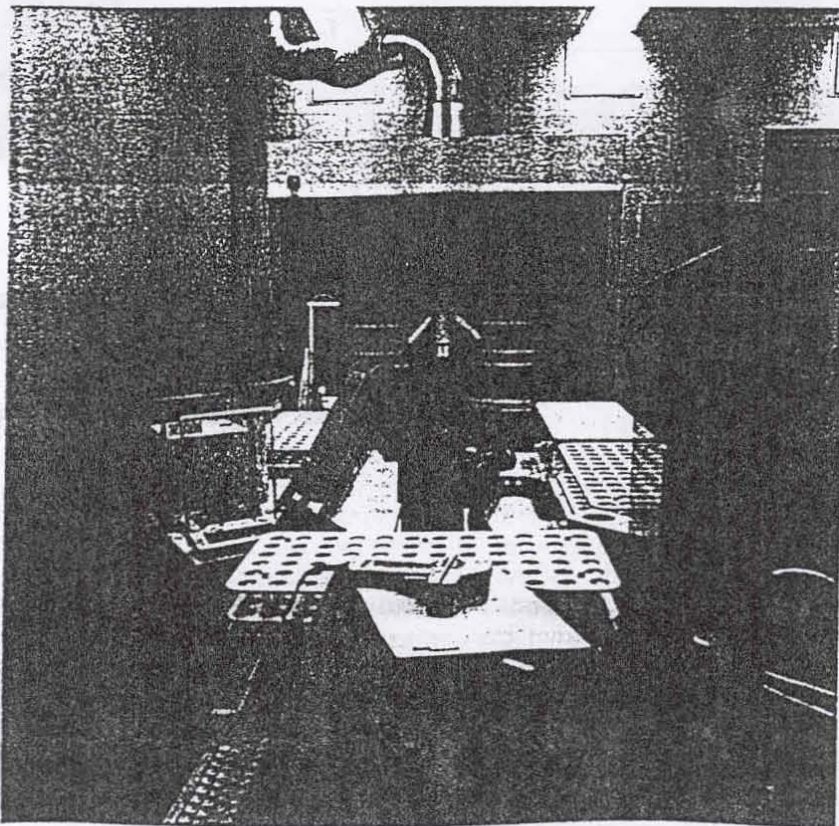
6-7 *figure* A robot arm used for acid digestion of samples prior to analysis by inductively coupled plasma-atomic emission spectroscopy (ICP-AES).
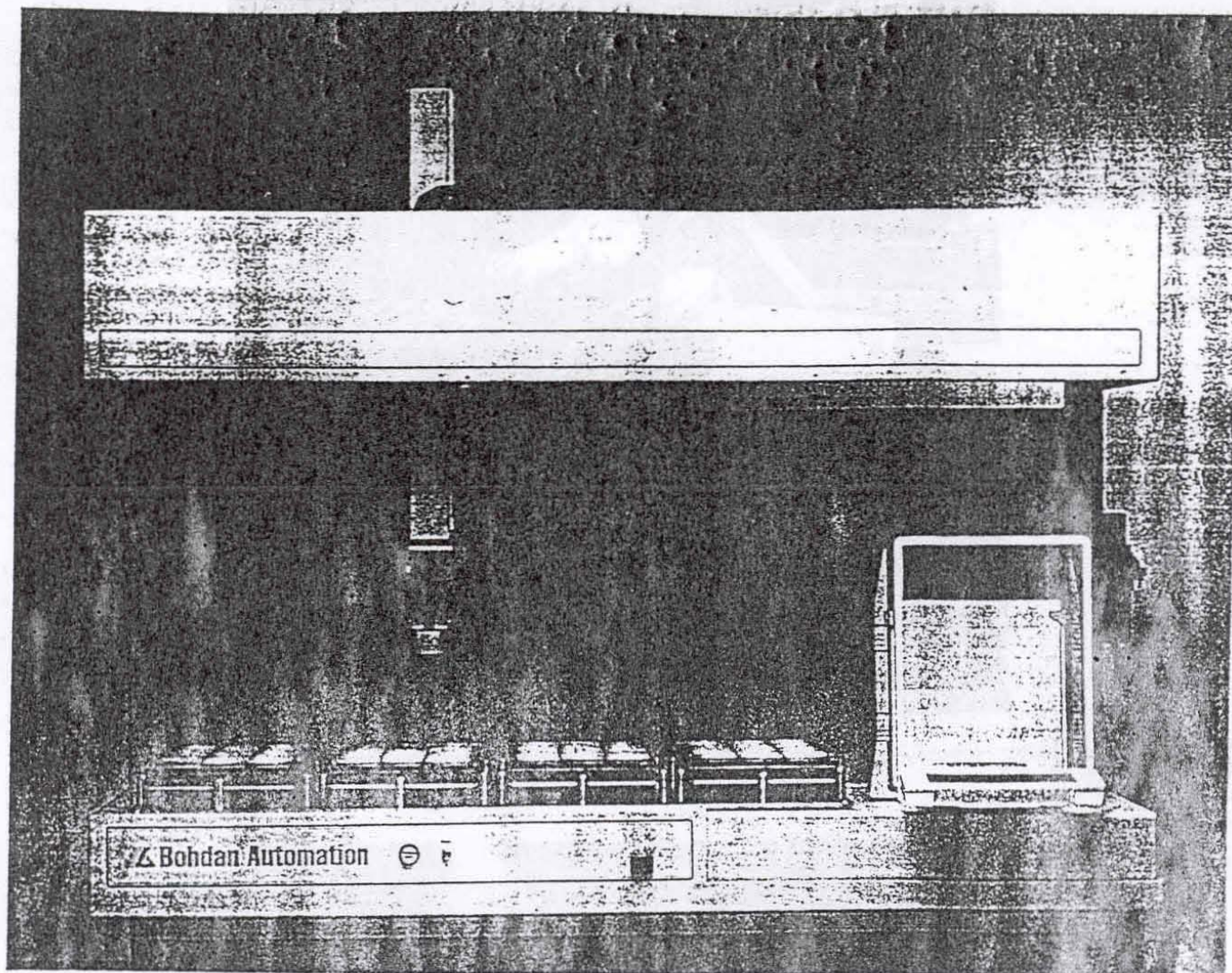
6-8 figure   Automated sample weighing station. (Courtesy of Bohdan Automation, Mundelein, IL.)

ing and selecting bits and pieces of this knowledge. When a particular problem needs to be solved, the expert system can rely on this knowledge to help provide a solution.

The use of expert systems with laboratory automation, especially robotics, takes the power of the computer one step further. Instead of merely controlling a robot or automated analyzer under a rigid program regimen, the expert system has the ability to modify the activity of the system. The knowledge that the expert system would use may simply be a list of rules for checking the types of analytical samples with available methods to help schedule the best way to run several analyses. Another possible knowledge base could help evaluate robotic sense inputs to develop a more accurate picture of the world around it. As the range of applications for LIMS and laboratory automation

increases the role of the expert system will become more evident.

6.4 SUMMARY

Significant progress has been made through the use of computers and automation over the last 20 years. This chapter details some of these recent advances in the food sciences with new applications for data acquisition and control as well as new laboratory robotics. Examples are given for several analytical methods used in food laboratories. The background and use of laboratory robotics are covered, with emphasis on systems integration and future trends in enhanced data management.

Most modern laboratory instrumentation is manu-
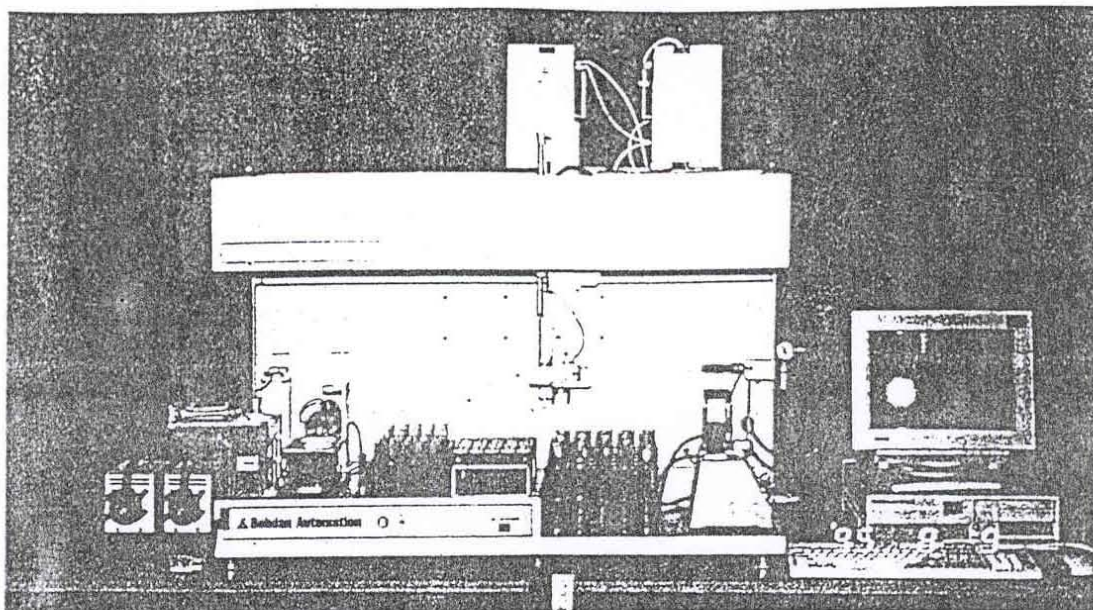
6-9 figure Chromatographic preparation workstation utilizing X–Y–Z manipulators. (Courtesy of Bohdan Automation, Mundelein, IL.)

factured with digital data-handling capabilities of some form. If an instrument is "computer compatible," the scientist possibly may need to acquire interfacing hardware, drivers, and software to capture and process the data. If a vendor does supply all these components, the chemist must find or develop the software to effectively interface or "computerize" the instrument.

6.5 STUDY QUESTIONS

1. Some advantages of automated data acquisition by computer might include less chance of errors in recording results, greater reproducibility of measurements, possibility for operations unattended by technician, ease in obtaining permanent records, ease in treating data with graphics, and utilization of report-generating programs. List others that would be of importance to you.

2. Some disadvantages of automated data acquisition by computer might include loss of understanding of the process when it is treated as a black box and the fact that reliance on a computer-controlled protocol may not facilitate insight into limitations of the information obtained. List others that would be of importance to you.

3. A vendor has just sold you a new automated balance. It has a built in RS-232c port and is claimed to be computer compatible. What additional items might be required to have a computer automate a series of weighings?

4. You read a report generated by a new computer-controlled infrared analyzer. The result you are checking shows "Fat content = 43.7146%." What are some potential limitations to this result? Is it wise to believe, without question, results printed by a computer?

5. Differentiate between the concepts of real-time and post-run data acquisition and analysis.

6. Suggest alternative steps for automating a robotic method for titratable acidity. List the laboratory devices required to perform the method and then suggest possible modifications to each device (e.g., specialized glassware) that would increase the efficiency of the automated method.

7. Review some analytical methods that would be good candidates for robotic automation. Give specific reasons for each; include human and environmental factors.

8. A large analytical laboratory will generally have a LIMS, or laboratory information management system, to store and recover analytical data. What other functions would be desirable in a food laboratory LIMS?

6.6 RESOURCE MATERIALS

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Chemical Composition and Characteristics of Foods

7

chapter

pH and Titratable Acidity

George D. Sadler and Patricia A. Murphy

7.1 INTRODUCTION

There are two interrelated concepts in food analysis that deal with acidity: pH and titratable acidity. Each of these quantities are analytically determined in separate ways and each has its own particular impact on food quality. Titratable acidity deals with measurement of the total acid concentration contained within a food. This quantity is determined by exhaustive titration of intrinsic acids with a standard base. Titratable acidity is a better predictor of an acid's impact on flavor than pH.

Total acidity does not tell the full story, however. Foods establish elaborate buffering systems that dictate how hydrogen ions (H^+), the fundamental unit of acidity, are expressed. Even in the absence of buffering, less than 3% of any food acid is ionized into H^+ and its anionic parent species (its conjugate base). This percentage is further suppressed by buffering. In aqueous solution, hydrogen ions combine with water to form hydronium ions, H_3O^+. The ability of a microorganism to grow in a specific food is an important example of a process that is more dependent on hydronium ion concentration than titratable acidity. The need to quantify only the free H_3O^+ concentration leads to the second major concept of acidity, that of pH. In nature, the H_3O^+ concentration can span a range of 14 orders of magnitudes. The term pH is a mathematical shorthand for expressing this broad continuum of H_3O^+ concentration in a concise and convenient notation. In contemporary food analysis, pH is usually determined instrumentally with a pH meter; however, chemical pH indicators also exist.

7.2 CALCULATION AND CONVERSION FOR NEUTRALIZATION REACTIONS

7.2.1 Concentration Units

This chapter deals with the theory and practical application of titratable acidity calculation and pH determination. To quantitatively measure components of foods, solutions must be prepared to accurate concentrations and diluted into the desired working range.

The terms used for concentration in food analysis should be reviewed. The most common concentration terms are given in Table 7-1. Molarity and normality are the most common SI (International Scientific) terms used in food analysis, but solutions also can be expressed as percentages. It is important that the analyst be able to convert between both systems.

Molarity (M) is a concentration unit representing the number of moles of the solute per liter of solution. Normality (N) is a concentration unit representing the number of equivalents (Eq) of a solute per liter of solution. In acid and base solutions, the normality represents the concentration or moles of H^+ or OH^- per liter that will be exchanged in a neutralization reaction

when taken to completion. For oxidation–reduction reagents, the normality represents the concentration or moles of electrons per liter to be exchanged when the reaction is taken to completion. The following are some examples of molarity versus normality (equivalents):

Acid–Base Reactions

1 M H_2SO_4 = 2 N H_2SO_4	2 equivalents of H^+ per mole of acid
1 M NaOH = 1 N NaOH	1 equivalent of OH^- per mole of base
1 M CH_3COOH = 1 N acetic acid	1 equivalent of H^+ per mole of acid
1 M $H_2C_4H_4O_5$ = 2 N malic acid	2 equivalents of H^+ per mole of acid

Oxidation–Reduction Reactions
e.g.

$$HSO^-_3 + I_2 + H_2O \rightleftharpoons SO_4^{2-} + 2I^- + 3H^+$$
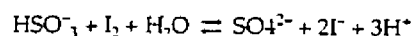
1 M I_2 = 2 N iodine	2 equivalents of electrons gained per mole of I_2
1 M HSO_3^- = 2 N bisulfite	2 equivalents of electrons lost per mole of bisulfite

Many analytical determinations in food analysis use the concept of equivalents to measure the amount of an unknown. Perhaps the most familiar of these are acid–base reactions in which hydrogen ions are exchanged and can be quantified through stoichiometric neutralization with a standard base. Acid–base reactions are used to determine nitrogen in the Kjeldahl protein determination (see Chapter 15), benzoic acid in sodas, and in determining percent titratable acidity. The concept of equivalents also is used in oxidation–reduction problems to quantify unknown analytes that are capable of direct electron transfer (see Chapter 10).

Equivalent weight can be defined as the molecular weight divided by the number of equivalents in the reactions. For example, the molecular weight of H_2SO_4 is 98.08 g. Since there are 2 equivalents per mole of H_2SO_4, the equivalent weight of H_2SO_4 is 49.04 g. Table 7-2 provides a list of molecular and equivalent weights for acids important in food analysis. In working with normality and milliliters, the term milliequivalents (mEq) is usually preferred. Milliequivalent weight is the equivalent weight divided by 1000.

Percentage concentrations are the mass amount of solute or analyte per 100 ml or 100 g of material. Percentage can be expressed for solutions or for solids and can be on a volume basis or mass basis. When the percentage becomes a number less than 1%, parts per million (ppm), parts per billion (ppb), and even parts per trillion (ppt) usually are preferred. If percentage is defined as the mass of the solute or analyte per mass (or volume) of sample × 100, then ppm is simply the same ratio of mass of solute per mass of sample × 1,000,000.

7-1
table   Concentration Expressions Terms

Unit	Symbol	Definition	Relationship
Molarity	M	Number of moles of solute per liter of solution	$M = \dfrac{moles}{liter}$
Normality	N	Number of equivalents of solute per liter of solution	$N = \dfrac{equivalents}{liter}$
Percent by weight (parts per hundred)	wt %	Ratio of weight of solute to weight of solute plus weight of solvent × 100	$wt = \dfrac{wt\ solute \times 100}{total\ wt}$
	wt/vol%	Ratio of weight of solute to total volume × 100	$wt\% = \dfrac{wt\ solute \times 100}{total\ volume}$
Percent by volume	vol%	Ratio of volume of solute to total volume	$vol\% = \dfrac{vol\ solute \times 100}{total\ volume}$
Parts per million	ppm	Ratio of solute (wt or vol) to total wt or vol × 1,000,000	$ppm = \dfrac{mg\ solute}{kg\ solution}$ $or = \dfrac{ng\ solute}{g\ solution}$ $or = \dfrac{mg\ solute}{liters\ solution}$ $or = \dfrac{ng\ solute}{ml\ solution}$
Parts per billion	ppb	Ratio of solute (wt or vol) to total wt or vol × 1,000,000,000	$ppb = \dfrac{ng\ solute}{liters\ solution}$ $or = \dfrac{ng\ solute}{kg}$ $or = \dfrac{fg\ solute}{ml}$ $or = \dfrac{fg\ solute}{g}$

7-2
table   Molecular and Equivalent Weights of Common Food Acids

Acid	Chemical Formula	Molecular Weight	Equivalents per Mole	Equivalent Weight
Citric (anhydrous)	$H_3C_6H_5O_7$	192.12	3	64.04
Citric (hydrous)	$H_3C_6H_5O_7 \cdot H_2O$	210.14	3	70.05
Acetic	$HC_2H_3O_2$	60.06	1	60.05
Lactic	$HC_3H_5O_3$	90.08	1	90.08
Malic	$H_2C_4H_4O_5$	134.09	2	67.05
Oxalic	$H_2C_2O_4$	90.04	2	45.02
Tartaric	$H_2C_4H_4O_6$	150.09	2	75.05
Ascorbic	$H_2C_6H_6O_6$	176.12	2	88.06
Hydrochloric	HCl	36.47	1	36.47
Sulfuric	H_2SO_4	98.08	2	49.04
Phosphoric	H_3PO_4	98.00	3	32.67
Potassium acid phthalate	$KHC_8H_4O_4$	204.22	1	204.22

7.2.2 Equation for Neutralization and Dilution

There are some general rules in evaluating equilibrium reactions that are helpful in most situations. At full neutralization the milliequivalents (mEq) of one reactant in the neutralization equals the milliequivalents of

the other reactant. This can be expressed mathematically as:

$$(ml\ of\ X)\ (N\ of\ X) = (ml\ of\ Y)\ (N\ of\ Y) \qquad [1]$$

Equation [1] also can be used to solve dilutions prob-

lems where X represents the stock solution and Y represents the working solution. When Equation [1] is used for dilution problems, any value of concentration (grams, moles, ppm, etc.) can be substituted for N. Units should be recorded with each number. Cancellation of units provides a quick check on proper setup of the problem. (See practice problems 1–8 at the end of Chapter 7.)

7.3 pH

7.3.1 Acid–Base Equilibria

The Bonsted–Lowry theory of neutralization is based upon the following definitions for acid and base:

Acid: A substance capable of donating protons. In food systems the only significant proton donor is the hydrogen ion.

Base: A substance capable of accepting protons.

Neutralization is the reaction of an acid with a base to form a salt as shown below:

$$HCl + NaOH \rightleftharpoons NaCl + H_2O \qquad [2]$$

Acids form hydrated protons called hydronium ions (H_3O^+) and bases form hydroxide ions (OH^-) in aqueous solutions:

$$H_3O^+ + OH^- \rightleftharpoons 2H_2O \qquad [3]$$
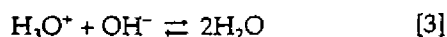
At any temperature, the product of the molar concentrations (moles/liter) of H_3O^+ and OH^- is a constant referred to as the ion product constant for water (K_w):

$$[H_3O^+][OH^-] = K_w \qquad [4]$$

K_w varies with the temperature. For example, at 25°C, $K_w = 1.04 \times 10^{-14}$ but at 100°C, $K_w = 58.2 \times 10^{-14}$.

The above concept of K_w leads to the question of what the concentrations of $[H_3O^+]$ and $[OH^-]$ are in pure water. Experimentation has revealed that the concentration of $[H_3O^+]$ is approximately 1.0×10^{-14} M, as is that of the $[OH^-]$ at 25°C. Because the concentrations of these ions are equal, pure water is referred to as being neutral.

Suppose that a drop of acid is added to pure water. The $[H_3O^+]$ concentration would increase. However, K_w would remain constant (1.0×10^{-14}), revealing a decrease in the $[OH^-]$ concentration. Conversely, if a drop of base is added to pure water, the $[H_3O^+]$ would decrease while the $[OH^-]$ would increase, maintaining the K_w at 1.0×10^{-14} at 25°C.

How did the term pH derive from the above considerations? In approaching the answer to this question, one must observe the concentrations of $[H_3O^+]$ and $[OH^-]$ in various foods, as shown in Table 7-3. The

numerical values found in Table 7-3 for $[H_3O^+]$ and $[OH^-]$ are bulky and led a Swedish chemist, S.L.P Sorensen, to develop the pH system in 1909.

pH is defined as the logarithm of the reciprocal of the hydrogen ion concentration. It also may be defined as the negative logarithm of the molar concentration of hydrogen ions. Thus, a $[H_3O^+]$ concentration of 1×10^{-6} is expressed simply as pH 6. The $[OH^-]$ concentration is expressed as pOH and would be pOH 8 in this case, as shown in Table 7.4.

While the use of pH notation is simpler from the numerical standpoint, it is a confusing concept in the minds of many students. One must remember that it is a logarithmic value and that a change in one pH unit is actually a 10-fold change in the concentration of $[H_3O^+]$. (See practice problems 9–12 at the end of Chapter 7.)

It is important to understand that pH and titratable acidity are not the same. Strong acids such as hydrochloric, sulfuric, and nitric acids are almost fully dissociated at pH 1. Only a small percentage of food acid molecules (citric, malic, acetic, tartaric, etc) dissociate in solution. This point may be illustrated by comparing the pH of 0.1 N solutions of hydrochloric and acetic acids.

$$HCl \rightleftharpoons H^+ + Cl^- \qquad [5]$$
$$CH_3COOH \rightleftharpoons H^+ + CH_3COO^- \qquad [6]$$
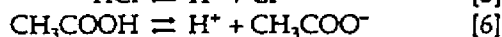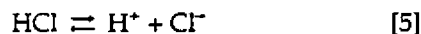
The HCl fully dissociates in solution to produce a pH of 1.02 at 25°C. By contrast, only about 1% of CH_3COOH is ionized at 25°C, producing a significantly lower pH of 2.89. The calculation and significance of partial dissociation on pH is presented in more detail in section 7.4.1.1.

7.3.2 pH Meter

7.3.2.1 Activity versus Concentration

In using pH electrodes, the concept of activity versus concentration must be considered. Activity is a measure of expressed chemical reactivity, while concentration is a measure of all forms (free and bound) of ions in solution. Due to interactions of ions between themselves and with the solvent, the effective concentration or activity is, in general, lower than the actual concentration, although activity and concentration tend to approach each other at infinite dilution. Activity and concentration are related by the following equation:

$$A = \gamma C \qquad [7]$$

where:

A = activity
γ = activity coefficient
C = concentration

| | **7-3** table | | **Concentrations of H_3O^+ and OH^- in Various Foods at 25°C** | | |

Food	$[H_3O^+]^1$	$[OH^-]^1$	K_w
Cola	2.24×10^{-3}	4.66×10^{-12}	1×10^{-14}
Grape juice	5.62×10^{-4}	1.78×10^{-11}	1×10^{-14}
SevenUp	3.55×10^{-4}	2.82×10^{-11}	1×10^{-14}
Schlitz beer	7.95×10^{-5}	1.26×10^{-10}	1×10^{-14}
Pure water	1.00×10^{-7}	1.00×10^{-7}	1×10^{-14}
Tap water	4.78×10^{-9}	2.09×10^{-6}	1×10^{-14}
Milk of magnesia	7.94×10^{-11}	1.26×10^{-4}	1×10^{-14}

From (12), used with permission. Copyright 1971 American Chemical Society.
^{1}Moles per liter.
Calculating the pH of the cola:
Step 1. Substitute the $[H^+]$ into the pH equation:
$pH = -log [H^+]$
$pH = -log (2.24 \times 10^{-3})$
Step 2. Separate 2.24×10^{-3} into two parts; determine the logarithm of each part:
$log 2.24 = 0.350$
$log 10^{-3} = -3$
Step 3. Add the two logs together since adding logs is equivalent to multiplying the two numbers:
$0.350 + (-3) = 2.65$
Step 4. Place the value into the pH equation:
$pH = -(-2.65)$
$pH = 2.65$

| | **7-4** table | | **Relationship of $[H^+]$ versus pH and $[OH^-]$ versus pOH at 25°C** |

$[H^+]^1$	pH	$[OH^-]^1$	pOH
1×10	0	1×10^{-14}	14
10^{-1}	1	10^{-13}	13
10^{-2}	2	10^{-12}	12
10^{-3}	3	10^{-11}	11
10^{-4}	4	10^{-10}	10
10^{-5}	5	10^{-9}	9
10^{-6}	6	10^{-8}	8
10^{-7}	7	10^{-7}	7
10^{-8}	8	10^{-6}	6
10^{-9}	9	10^{-5}	5
10^{-10}	10	10^{-4}	4
10^{-11}	11	10^{-3}	3
10^{-12}	12	10^{-2}	2
10^{-13}	13	10^{-1}	1
10^{-14}	14	10^{0}	0

From (12), used with permission. Copyright 1971 American Chemical Society.
^{1}Moles per liter. Note that the product of $[H^+][OH^-]$ is always 1×10^{-14}.
Calculation of $[H^+]$ of a beer with pH 4.30:
Step 1. Substitute numbers into the pH equation:
$pH = -log [H^+]$
$4.30 = -log [H^+]$
$-4.30 = log [H^+]$
Step 2. Divide the -4.30 into two parts so that the first part contains the decimal places and second part the whole number:
$-4.30 = 0.70 - 5 = log [H^+]$
Step 3. Find the antilogs:
antilog of $0.70 = 5.0$
antilog of $-5 = 10^{-5}$
Step 4. Multiply the two antilogs to get $[H^+]$:
$5 \times 10^{-5} = [H^+]$
$[H^+] = 5 \times 10^{-5} M$

The activity coefficient is a function of ionic strength. Ionic strength is a function of the concentration of, and the charge on, all ions in solution. Activity issues can become significant for hydronium ions below pH 1 and for hydroxyl ions at pH 13 and above.

7.3.2.2 General Principles

The pH meter is a good example of a potentiometer (a device that measures voltage at infinitesimal current flow). The basic principle of potentiometry (an electrochemical method of voltammetry at zero current) involves the use of an electrolytic cell composed of two electrodes dipped into a test solution. A voltage develops, which is related to the ionic concentration of the solution. Since the presence of current could alter the concentration of surrounding ions or produce irreversible reactions, this voltage is measured under conditions such that infinitesimal current (10^{-12} amperes or less) is drawn.

Four major parts of the pH system are needed: (1) reference electrode, (2) indicator electrode (pH sensitive), (3) voltmeter or amplifier that is capable of measuring small voltage differences in a circuit of very high resistance, and (4) the sample being analyzed (Fig. 7-1).

One notes that there are two electrodes involved in the measurement. Each of these electrodes is designed carefully to produce a constant, reproducible potential. Therefore, in the absence of other ions, the potential difference between the two electrodes is fixed and easily calculated. However, H_3O^+ ions in solution con-
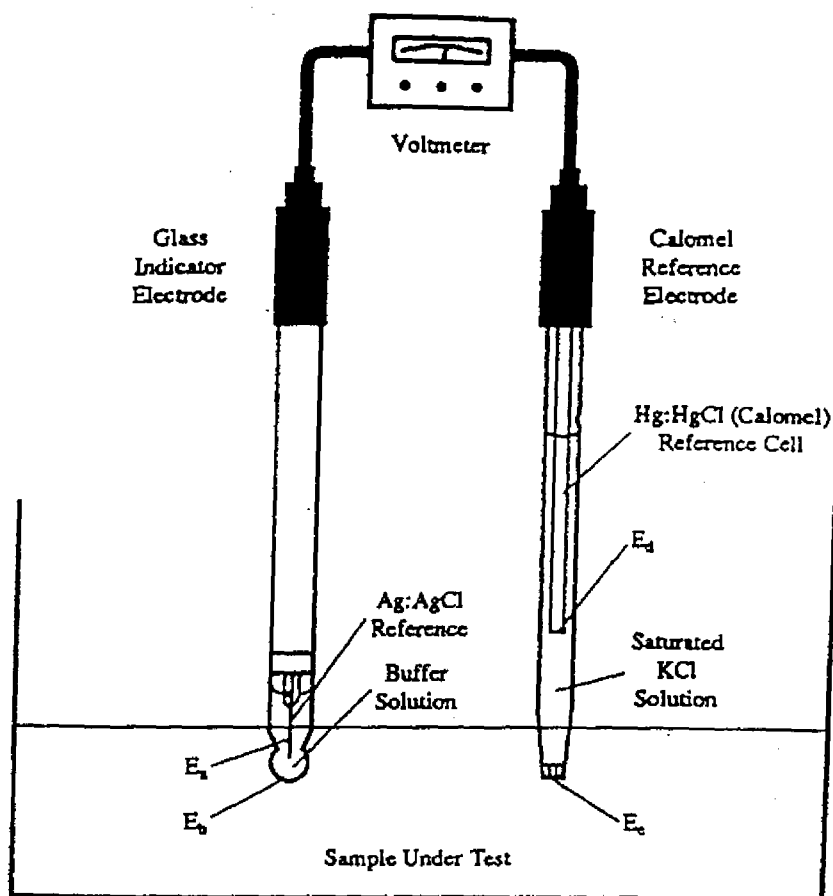
7-1 figure

The measuring circuit of the potentiometric system. E_a: contact potential between Ag:AgCl electrode and inner liquid. E_a is independent of pH of the test solution but is temperature dependent. E_b: potential developed at the pH-sensitive glass membrane. E_b varies with the pH of the test solution and also with temperature. In addition to this potential the glass electrode also develops an asymmetry potential, which depends upon the composition and shape of the glass membrane. It also changes as the electrode ages. E_c: diffusion potential between saturated KCl solution and test sample. E_c is essentially independent of the solution under test. E_d: contact potential between calomel portion of electrode and KCl salt bridge. E_d is independent of the solution under test but is temperature dependent. [From (3), used with permission.]

tribute a new potential across an ion-selective glass membrane built into the indicating electrode. This alters the potential difference between the two electrodes in a way that is proportional to H_3O^+ concentration. The new potential resulting from the combination of all individual potentials is called the electrode potential and is readily convertible into pH readings.

Hydrogen ion concentration (or more accurately, activity) is determined by the voltage that develops between the two electrodes. The Nernst equation relates the electrode response to the activity where:

$$E = E° + 2.303 \frac{RT}{NF} \log A \qquad [8]$$

where:

E = measured electrode potential

$E°$ = standard electrode potential, a constant repre-

senting the sum of the individual potentials in the system at a standard temperature, ion concentration, and electrode composition

R = universal gas constant, 8.313 joules/degree/g mole wt

F = Faraday constant, 96,490 coulombs per g equiv wt

T = absolute temperature (Kelvin)

N = number of charges on the ion

A = activity of the ion being measured

For monovalent ions (such as the hydronium ion) at 25°C, the relationship of 2.303 RT/F is calculated to be 0.0591, as follows:

$$\frac{2.303 \times 8.316 \times 298}{96,490} = 0.0591 \qquad [9]$$

Thus, voltage produced by the electrode system is a

linear function of the pH, the electrode potential being essentially + 59 millivolts (0.059 volts) for each change of one pH unit. At neutrality (pH 7), the electrode potential is zero millivolts. At pH 6, the electrode potential is +60 millivolts, while at pH 4, the electrode potential is +180 millivolts. Conversely, at pH 8, the electrode potential is -60 millivolts.

It must be emphasized that the above relationship between millivolts and pH exists only at 25°C, and changes in temperature will erroneously alter the pH reading. For example, at 0°C, the electrode potential is 54 millivolts, while at 100°C it is 70 millivolts. Modern pH meters have a sensitive attenuator (temperature compensator) built into them in order to account for this effect of temperature.

7.3.2.3 Reference Electrode

The reference electrode is needed to complete the circuit in the pH system. This half cell is one of the most troublesome parts of the pH meter. Problems in obtaining pH measurements are often traced to a faulty reference electrode.

The saturated calomel electrode (Fig. 7-1) is the most common reference electrode. It is based upon the following reversible reaction:

$$Hg_2Cl_2 + 2e^- \rightleftharpoons 2Hg + 2Cl^- \qquad [10]$$
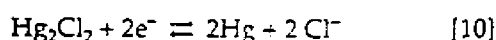
The $E_{0.25°C}$ for the saturated KCl salt bridge is +0.2444 volts versus a standard hydrogen electrode; the Nernst equation for the reaction is as follows:

$$E = E_0 - 0.059/2 \log [Cl^-]^2 \qquad [11]$$

Thus, one observes that the potential is dependent upon the chloride ion concentration, which is easily regulated by the use of saturated KCl solution in the electrode.

A calomel reference electrode has three principal parts: (1) a platinum wire covered with a mixture of calomel (Hg_2Cl_2), (2) a filling solution (saturated KCl), and (3) a permeable junction through which the filling solution slowly migrates into the sample being measured. Junctions are made of ceramic or fibrous material. These junctions tend to clog up, causing a slow, unstable response and inaccurate results.

A less widely used reference electrode is the silver–silver chloride electrode. Because the calomel electrode is unstable at high temperatures (80°C) or in strongly basic samples (pH > 9), a silver–silver chloride electrode must be used for such application. It is a very reproducible electrode based upon the following reaction:

$$AgCl(s) + e^- \rightleftharpoons Ag(s) + Cl^- \qquad [12]$$
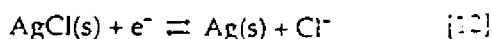
The internal element is a silver-coated platinum wire, the surface silver being convered to silver chloride by hydrolysis in hydrochloric acid. The filling solution is a mixture of 4 M KCl, saturated with AgCl that is used to prevent the AgCl surface of the internal element from dissolving. The permeable junction is usually of the porous ceramic type. Because of the relative insolubility of AgCl, this electrode tends to clog more readily than the calomel reference electrode. However, it is possible to obtain a double-junction electrode in which a separate inner body holds the Ag/AgCl internal element electrolyte, and ceramic junction. An outer body containing a second electrolyte and junction isolates the inner body from the sample.

7.3.2.4 Indicator Electrode

The indicator electrode most commonly used in measuring pH today is referred to as the glass electrode. Prior to its development, the hydrogen electrode and the quinhydrone electrode were used.

The history of the glass electrode goes back to 1875, when it was suggested by Lord Kelvin that glass was an electrical conductor. Cremer discovered the glass electrode potential 30 years later when he observed that a thin glass membrane placed between two aqueous solutions exhibited an electrical potential sensitive to changes in acidity. Subsequently, the reaction was shown to be dependent upon the hydrogen ion concentration. These observations were of great importance in the development of the pH meter.

What is the design of the glass electrode? This electrode (Fig. 7-1) also has three principal parts: (1) a silver–silver chloride electrode with a mercury connection that is needed as a lead to the potentiometer; (2) a buffer solution consisting of 0.01 N HCl, 0.09 N KCl, and acetate buffer used to maintain a constant pH (E_s); and (3) a small pH-sensitive glass membrane for which the potential (E_0) varies with the pH of the test solution. In using the glass electrode as an indicator electrode in pH measurements, the measured potential (measured against the calomel electrode) is directly proportional to the pH as discussed earlier, $E = E_0 + 0.059$ pH.

Conventional glass electrodes are suitable for measuring pH in the range of pH 1–9. However, this electrode is sensitive to higher pH, especially in the presence of sodium ions. Thus, equipment manufacturers have developed modern glass electrodes that are usable over the entire pH range of 0–14 and feature a very low sodium ion error, such as <0.01 pH at 25°C.

7.3.2.5 Combination Electrodes

Today, most food analysis laboratories use combination electrodes that combine both the pH and reference

electrodes along with the temperature sensing probe in a single unit or probe. These combination electrodes are available in many sizes and shapes from very small microprobes to flat surface probes, from all glass to plastic, and from exposed electrode tip to jacketed electrode tips to prevent glass tip breakage. Microprobes may be used to measure pH of very small systems such as inside a cell or a solution on a microscope slide. Flat surface electrode probes can be used to measure pH of semisolid and high viscosity substances such as meat, cheese, and agar plates and small volumes as low as 10 µl.

7.3.2.6 Guidelines for Use of pH Meter

It is very important that the pH meter be operated and maintained properly. One should always follow the specific instructions provided by the manufacturer. For maximum accuracy, the meter should be standardized using two buffers (two-point calibration). Select two buffers of pH values about 3 pH units apart, bracketing that of the anticipated sample pH. The three standardization buffers used most widely in laboratories are a pH 4.0 buffer, a pH 7.0 buffer, and a pH 9.0 buffer (at 25°C). These are the typical pink, yellow, and blue solutions found adjacent to pH meters in many laboratories.

When standardizing the pH electrode, follow manufacturer's instructions for one-point calibration; rinse thoroughly with distilled water and blot dry. Immerse electrode in the second buffer (pH 4, for example) and perform a second standardization. This time, the pH meter slope control is used to adjust the reading to the correct value of the second buffer. Repeat these two steps, if necessary, until a value within 0.1 pH unit of the correct value of the second buffer is displayed. If this cannot be achieved, the instrument is not in good working condition. Electrodes should be checked, remembering that the reference electrode is more likely in need of attention. One should always follow the electrode manufacturer's specific directions for storage of a pH electrode. In this way, the pH meter is always ready to be used and the life of the electrodes is prolonged. One precaution that should be followed pertains to a calomel reference electrode. The storage solution level always should be at least 2 cm below the saturated KCl solution level in the electrode in order to prevent diffusion of storage solution into the electrode (Fig. 7-2).

7.4 TITRATABLE ACIDITY

7.4.1 General Considerations

pH is used to determine the endpoint of acid–base titration. This can be achieved directly with a pH
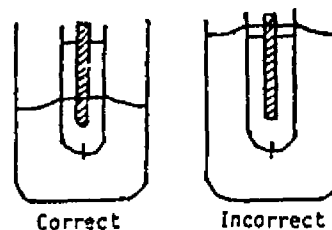
Correct   Incorrect

7-2 figure

Correct and incorrect depth of calomel electrodes in solutions. [Reprinted with permission from (12). Copyright 1971 American Chemical Society.]

meter, but more commonly an indicator dye is used. In some cases, the way pH changes during titration can lead to subtle problems. Some background in acid theory is necessary to fully understand titration and to appreciate the occasional problems that might arise.

7.4.1.1 Buffering

Although pH can hypothetically range from –1 to 14, pH readings below 1 are difficult to obtain. This is due to incomplete dissociation of hydrogen ions at high acid concentrations. At 0.1 N, strong acids are assumed to be fully disassociated. Therefore, fully disassociated acid is always present when a strong base is used to titrate a strong acid; and the pH at any point in the titration is equal to the hydrogen ion concentration of the remaining acid (Fig. 7-3).

mL 0.1 NaOH
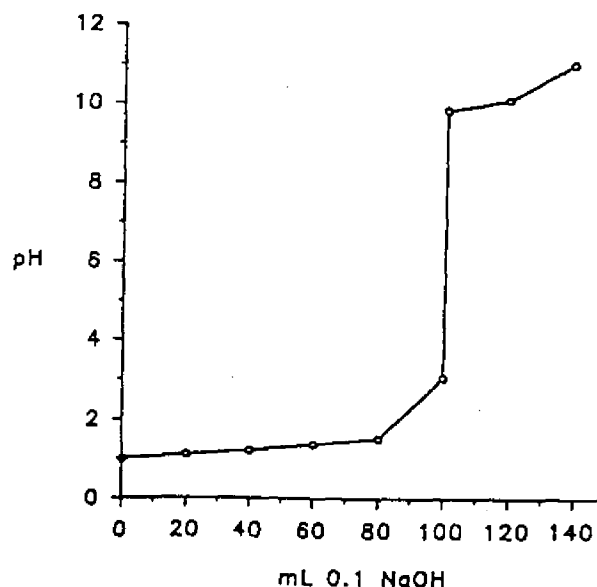
7-3 figure

Titration of a strong acid with strong base. The pH at any point in the titration is dictated by the hydrogen ion concentration of the acid remaining after partial neutralization with base.

All food acids are weak acids. Less than 3% of their ionizable hydrogens are disassociated from the parent molecule. When free hydrogen ions are removed through titration, new hydrogen ions can arise from other previously undisassociated parent molecules. This tends to cushion the solution from abrupt changes in pH. This property of a solution to resist change in pH is termed buffering. Buffering occurs in foods whenever a weak acid and its salt are present in the same medium. Due to buffering, a graph of pH versus titrant is more complex for weak acids than strong acids. However, this relationship can be predicted by the Henderson–Hasselbalch equation.

$$pH = pK_a + \log \frac{[A^-]}{[HA]} \qquad [13]$$

[HA] represents the concentration of undissociated acid. [A⁻] represents the concentration of its salt, also known as the conjugated base. The conjugated base is equal in concentration to the conjugated acid [H₃O⁺]. The pK_a is the pH at which equal quantities of undissociated acid and conjugated base are present. The equation indicates that maximum buffering capacity will exist when the pH equals the pK_a. A graph showing the titration of 0.1 N acetic acid with 0.1 N NaOH illustrates this point (Fig. 7-4).

Di- and triprotic acids will have two and three buffering regions, respectively. A pH versus titrant graph of citric acid is given in Fig. 7-5. If the pK_a steps

7-5
figure

Titration of a weak polyprotic acid with a strong base. Buffering regions are established around each pK_a. The Henderson–Hasselbalch equation can predict the pH for each pK_a value if pK_a steps are separated by more than three units. However, complex transition mixtures between pK_a steps make simple calculations of transition pH values impossible.

in polyprotic acids differ by three or more pK_a units, then the Henderson–Hasselbalch equation can predict the plateau corresponding to each step. However, the transition region between steps is complicated by the presence of protons and conjugate bases arising from other disassociation state(s). Consequently, the Henderson–Hasselbalch equation breaks down near the equivalence point between two pK_a steps. However, the pH at the equivalence point is easily calculated. The pH is simply $(pK_a1 + pK_a2)/2$. Table 7-5 lists pK_a values of acids important in food analysis.

7-4
figure

Titration of a weak monoprotic acid with a strong base. A buffering region is established around the pK_a (4.82). The pH at any point is described by the Henderson–Hasselbalch equation.

7-5
table

pK_a Values for Some Acids Important in Food Analysis

Acid	pK_a1	pK_a2	pK_a3
Oxalic	1.19	4.21	—
Phosphoric	2.12	7.21	12.30
Tartaric	3.02	4.54	—
Malic	3.40	5.05	—
Citric	3.06	4.74	5.40
Lactic	3.86	—	—
Ascorbic	4.10	11.79	—
Acetic	4.76	—	—
Potassium acid phthalate	5.40	—	—
Carbonic	6.10	10.25	—

7.4.1.2 Potentiometric Titration

At the equivalence point in a titration, the number of acid equivalents exactly equals the number of base equivalents, and total acid neutralization is achieved. As the equivalence point is approached, the denominator [HA] in the Henderson-Hasselbalch equation becomes insignificantly small and the quotient [A⁻]/[HA] increases exponentially. As a result, the solution pH rapidly increases and ultimately approaches the pH of the titrant. The exact equivalent point is the halfway mark on this slope of abrupt pH increase. The use of a pH meter to identify the endpoint is called the potentiometric method for determining titratable acidity. The advantage of determining the equivalence point potentiometrically is that the precise equivalence point is identified. Since a rapid change in pH (and not some final pH value per se) signals the end of titration, accurate calibration of the pH meter is not even essential. However, in order to identify the equivalence point, a careful record of pH versus titrant must be kept. This and the physical constraints of pH probes and slow response with some electrodes make the potentiometric approach somewhat cumbersome.

7.4.1.3 Indicators

For simplicity in routine work, an indicator solution is often used to approximate the equivalence point. This approach tends to overshoot the equivalence point by a small amount. When indicators are used, the term endpoint or colorimetric endpoint is substituted for equivalence point. This emphasizes that the resulting values are approximate and dependent on the specific indicator. Phenolphthalein is the most common indicator for food use. It changes from clear to red in the pH region 8.0 to 9.6. Significant color change is usually present by pH 8.2. This pH is termed the phenolphthalein endpoint.

A review of pK_a values in Table 7-5 indicates that naturally occurring food acids do not buffer in the region of the phenolphthalein endpoint. However, phosphoric acid (used as an acidulant in some soft drinks) and carbonic acid (carbon dioxide in aqueous solution) do buffer at this pH. Consequently, taking the solution from the true equivalence point to the endpoint may require a large amount of titrant for these acids. Indistinct endpoints and erroneously large titration values may result. When these acids are titrated, potentiometric analysis is usually preferred. Interference by CO_2 can be removed by boiling the sample and titrating the remaining acidity to a phenolphthalein endpoint.

Deeply colored samples also present a problem for endpoint indicators. When colored solutions obscure the endpoint, a potentiometric method is normally used. For routine work, pH versus titrant data are not collected. Samples are simply titrated to pH 8.2 (the phenolphthalein endpoint). Even though this is a potentiometric method, the resulting value is an endpoint and not the true equivalence point since it simply reflects the pH value for the phenolphthalein endpoint.

A pH of 7 may seem to be a better target for a potentiometric endpoint than 8.2. This pH, after all, marks the point of true neutrality on the pH scale. However, once all acid has been neutralized, the conjugate base remains. As a result, the pH at the equivalence point is slightly greater than 7. Confusion also might arise if pH 7 was the target for colored samples and pH 8.2 was the target for noncolored samples.

Dilute acid solutions (e.g., vegetable extracts) require dilute solutions of standard base for optimal accuracy in titration. However, a significant volume of dilute alkali may be required to take a titration from the equivalence point to pH 8.2. Bromthymol blue is used sometimes as an alternative indicator in low acid situations. It changes from yellow to blue in the pH range 6.0–7.6. The endpoint is usually a distinct green. However, endpoint identification is somewhat more subjective than the phenolphthalein endpoint.

Indicator solutions rarely contain over a few tenths percent dye (wt/vol). All indicators are either weak acids or weak bases that tend to buffer in the region of their color change. In excessive amounts, they can influence the titration by conferring their own acid/base character to the sample under analysis. Indicator solutions should be held to the minimum necessary to impart effective color. Typically, two to three drops of indicator are added to the solution to be titrated. The lower the indicator concentration, the sharper will be the endpoint.

In acetic acid fermentations, it is sometimes desirable to know how much acidity comes from the acetic acid and how much is contributed naturally by other acids in the product. This can be achieved by first performing an initial titration to measure total acidity. The acetic acid is then boiled off, the solution is allowed to cool, and a second titration is performed to determine the fixed acidity. The difference between fixed and total acidity is the volatile acidity. A similar practice is used sometimes in the brewing industry to separate acidity due to dissolved CO_2 from fixed acids. Fixed acids are titrated after CO_2 is removed by low heat (40°C) and gentle agitation.

7.4.2 Preparation of Reagents

7.4.2.1 Standard Alkali

Sodium hydroxide (NaOH) is the most commonly used base in titratable acidity determinations. In some ways, it appears to be a poor candidate for a standard

base. Reagent grade NaOH is very hygroscopic and often contains significant quantities of insoluble sodium carbonate (Na_2CO_3). Consequently, the normality of working solutions is not precise, but must be standardized against an acid of known normality. However, economy, availability, and long tradition of use for NaOH outweigh these shortcomings. Working solutions are normally made from a stock solution containing 50% sodium hydroxide in water (wt/vol). Sodium carbonate is essentially insoluble in concentrated alkali and gradually precipitates out of solution over the first 10 days of storage.

NaOH can react with dissolved and atmospheric CO_2 to produce new Na_2CO_3. This reduces alkalinity and sets up a carbonate buffer that can obscure the true endpoint of a titration. Therefore, CO_2 should be removed from water prior to making the stock solution. This can be achieved by purging water with CO_2-free gas for 24 hours or by boiling distilled water for 20 min and allowing it to cool before use. During cooling and long-term storage, air (with accompanying CO_2) will be drawn back into the container. Carbon dioxide can be stripped from reentering air with a soda-lime (20% NaOH, 65% CaO, 15% H_2O) or ascarite trap (NaOH impregnated asbestos). Air passed through these traps also can be used as purge gas to produce CO_2-free water.

Stock alkali solution of 50% in water is approximately 18 N. A working solution is made by diluting stock solution with CO_2-free water. There is no ideal container for strong alkali solutions. Glass and plastic are both used, but each has its drawbacks. If a glass container is used it should be closed with a rubber or thick plastic closure. Glass closures should be avoided since, over time, strong alkali dissolves glass, resulting in permanent fusion of the contact surfaces. Reaction with glass also lowers the normality of the alkali. These liabilities also are relevant to long-term storage of alkali in burettes. NaOH has a low surface tension. This predisposes to leakage around the stopcock. Stopcock leakage during titration will produce erroneously high acid values. Slow evaporation of titrating solution from the stopcock valve during long periods of non-use also creates a localized region of high pH with ensuing opportunities for fusion between the stopcock and burette body. After periods of non-use, burettes should be emptied, cleaned, and refilled with fresh working solution.

Long-term storage of alkali in plastic containers also requires special vigilance because CO_2 permeates freely through most common plastics. Despite this shortcoming, plastic containers are usually preferred for long-term storage of stock alkali solutions. Whether glass or plastic is used for storage, working solutions should be restandardized weekly to correct for alkalinity losses arising from interactions with glass and CO_2.

7.4.2.2 Standard Acid

The impurities and hygroscopic nature of NaOH make it unsuitable as a primary standard. Therefore, NaOH titrating solutions must be standardized against a standard acid. Potassium acid phthalate (KHP) is commonly used for this purpose.

KHP's single ionizable hydrogen ($pK_a = 5.4$) provides very little buffering at pH 8.2. It can be manufactured in very pure form, it is relatively nonhygroscopic, and it can be dried at 120°C without decomposition or volatilization. Its high molecular weight also favors accurate weighing.

KHP should be dried for 2 hours at 120°C and allowed to cool to room temperature in a desiccator immediately prior to use. An accurately measured quantity of KHP solution is titrated with a base of unknown normality. The base is always the titrant. CO_2 is relatively insoluble in acidic solutions. Consequently, stirring an acid sample to assist in mixing will not significantly alter the accuracy of the titration.

7.4.3 Sample Analysis

A number of official methods exist for determining titratable acidity in various foods (AOAC International, 1995). However, determining titratable acidity on most samples is relatively routine and various procedures share many common steps. An aliquot of sample (often 10 ml) is titrated with a standard alkali solution (often 0.1 N NaOH) to a phenolphthalein endpoint. Potentiometric endpoint determination is used when sample pigment makes use of a color indicator impractical.

Typical titration setups are illustrated in Fig 7-6 for potentiometric and colorimetric endpoints. Erlenmeyer flasks are usually preferred for samples when endpoint indicators are used. A magnetic stirring bar may be used, but mixing the sample with hand swirling is usually adequate. When hand mixing is used the sample flask is swirled with the right hand. The stopcock is positioned on the right side. Four fingers on the left hand are placed behind the stopcock valve and the thumb is placed on the front of the valve. Titrant is dispensed at a slow, uniform rate until the endpoint is approached and then added dropwise until the endpoint does not fade after standing for some predetermined period of time, usually 5–10 sec.

The bulkiness of the pH electrode usually demands that beakers be used instead of Erlenmeyer flasks when samples are analyzed potentiometrically. Mixing is almost always achieved through magnetic stirring, and loss of sample through splashing is more likely with beakers than Erlenmeyer flasks. Otherwise, titration practices are identical to those described previously for indicator solutions.

Titratable Acidity Apparatus

1. Burette

2. Burette Clamp

3. Clamp
   Support

4. Magnetic Stirring
   Plate

5. Stopcock

6. pH Meter

7. Combination pH
   Probe

Potentiometric Titration   Colorimetric Titration
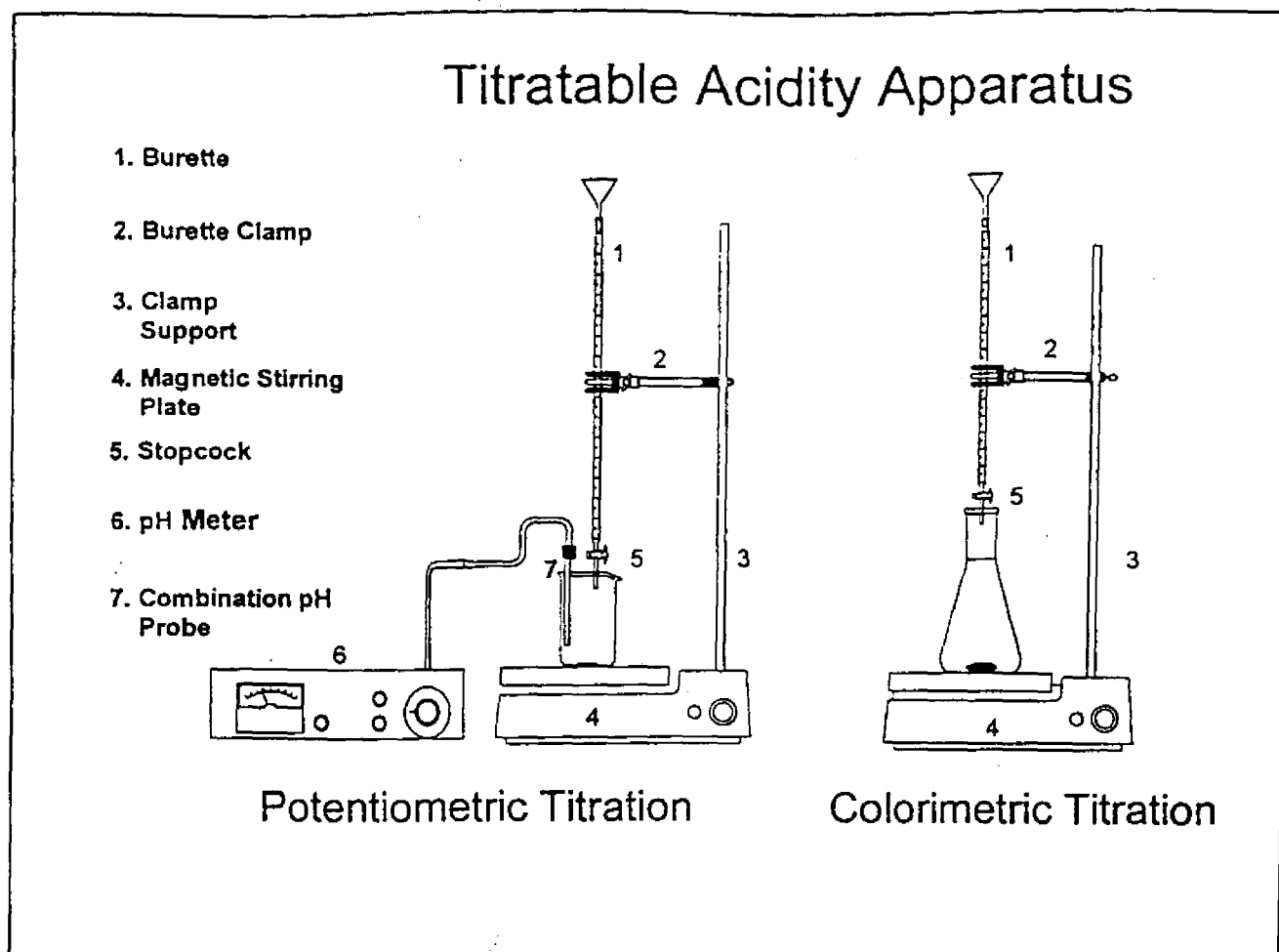
7-6
figure   Titratable acidity apparatus.

Problems may arise when concentrates, gels, or particulate-containing samples are titrated. These matrices prevent rapid diffusion of acid from densely packed portions of sample material. This slow diffusion process results in a fading endpoint. Concentrates can simply be diluted with CO_2-free water. Titration then is performed and the original acid content calculated from dilution data. Starch and similar weak gels often can be mixed with CO_2-free water, stirred vigorously, and titrated in a manner similar to concentrates. However, some pectin and food gum gels require mixing in a blender to adequately disrupt the gel matrix. Thick foams are occasionally formed in mixing. Antifoam or vacuum can be used to break the foams.

Immediately following processing, the pH values of particulate samples often vary from one particulate piece to another. Acid equilibration throughout the entire mass may require several months. As a result, particulate-containing foods should be comminuted finely in a blender before titrating. The comminuting process may incorporate large quantities of air. Air entrapment makes the accuracy of volumetric measurements questionable. Aliquots often are weighed when air incorporation may be a problem.

7.4.4 Acid Content in Food

Most foods are as chemically complex as life itself. As such, they contain the full complement of Krebs cycle acids (and their derivatives), fatty acids, and amino acids. Theoretically, all of these contribute to titratable acidity. Routine titration cannot differentiate between individual acids. Therefore, titratable acidity is usually stated in terms of the predominant acid. For most foods this is unambiguous. In some cases, two acids are present in large concentrations and the predominant acid may change with maturity. In grapes, malic acid often predominates prior to maturity while tartaric

acid typically predominates in the ripe fruit. A similar phenomenon is observed with malic and citric acids in pears. Fortunately, the equivalent weights of common food acids are fairly similar. Therefore, percent titratable acidity is not substantially affected by mixed predominance or incorrect selection of the predominant acid.

The range of acid concentrations in foods is very broad. Acids can exist at levels below detection limits or they can be the preeminent substance present in the food. The contribution of acids to food flavor and quality is not told by acid content alone. The tartness of acids is reduced by sugars. Consequently, the Brix/acid ratio (often simply called ratio) is usually a better predictor of an acid's flavor impact than Brix or acid alone. Acids tend to decrease with the maturity of fruit while sugar content increases. Therefore, the Brix/acid ratio often is used as an index of fruit maturity. The ratio also can be affected by climate, variety, and horticultural practices. Table 7-6 gives typical acid composition and sugar levels for many commercially important fruits at maturity. Citric and malic acids are the most common acids in fruits and most vegetables; however, leafy vegetables also may contain significant quantities of oxalic acid. Lactic acid is the most important acid in dairy foods for which titratable acidity is commonly used to monitor the progress of lactic acid fermentations in cheese and yogurt production (Marshall, 1992).

Organic acids contribute to the refractometer reading of soluble solids. When foods are sold on the basis of pounds solids, Brix readings are sometimes corrected for acid content. For citric acid, 0.20° Brix are added for each percent titratable acidity.

7.4.5 Other Methods

High performance liquid chromatography (HPLC) and electrochemistry both have been used to measure acids in food samples. Both methods allow identification of specific acids. HPLC uses refractive index, ultraviolet, or for some acids electrochemical detection. Ascorbic acid has strong electrochemical signature and significant absorbance at 265 nm. Significant absorbance of other prominent acids does not occur until 200 nm or below.

Many acids can be measured with such electrochemical techniques as voltammetry and polarography. In ideal cases, the sensitivity and selectivity of electrochemical methods are exceptional. However, interfering compounds often reduce the practicality of electrochemical approaches.

Unlike titration, chromatographic and electrochemical techniques do not differentiate between an acid and its conjugate base. Both species inevitably exist side by side as part of the inherent food–buffer system. As a result, acids determined by instrumental methods may be 50% higher than values determined by titration. It follows that Brix/acid ratios can be based only on acid values determined by titration.

7.5 SUMMARY

Organic acids have a pronounced impact on food flavor and quality. Unlike strong acids which are fully dissociated, food acids are only partially ionized. Some properties of foods are affected only by this ionized fraction of acid molecules while other properties are affected by the total acid content. It is impractical to quantify only free hydronium ions in solution by chemical methods. Once the free ions are removed by chemical reaction, others arise from previously undissociated molecules. Indicator dyes, which change color depending on the hydronium ion environment, exist but they only identify when a certain pH threshold has been achieved and do not stoichiometrically quantify free hydronium ions. The best that can be done is to identify the secondary effect of the hydronium ion environment on some property of the system such as the color of the indicator dyes or the electrochemical potential of the medium. The pH meter measures the change in electrochemical potential established by the hydronium ion across a semipermeable glass membrane on an indicator electrode. The shift in the indicator electrode potential is indexed against the potential of a reference electrode. The difference in millivolt reading between the two electrodes can be converted into pH using the Nerst equation. The hydronium ion concentration can be back calculated from pH using the original definition of pH as the negative log of hydrogen ion concentration. Buffer solutions of any

| | Acid Composition and °Brix of Some Commercially Important Fruits |

Table 7-6

Fruit	Principal Acid	Typical Percent Acid	Typical °Brix
Apples	Malic	0.27–1.02	9.12–13.5
Bananas	Malic/citric (3:1)	0.25	16.5–19.5
Cherries	Malic	0.47–1.86	13.4–18.0
Cranberries	Citric	0.9–1.36	
	Malic	0.70–0.98	12.9–14.2
Grapefruit	Citric	0.64–2.10	7–10
Grapes	Tartaric/malic (3:2)	0.64–1.16	13.3–14.4
Lemons	Citric	4.2–8.33	7.1–11.9
Limes	Citric	4.9–8.3	8.3–14.1
Oranges	Citric	0.68–1.20	9–14
Peaches	Citric	1–2	11.8–12.3
Pears	Malic/citric	0.34–0.45	11–12.3
Pineapples	Citric	0.78–0.84	12.3–16.8
Raspberries	Citric	1.57–2.23	9–11.1
Strawberries	Citric	0.95–1.18	8–10.1
Tomatoes	Citric	0.2–0.6	4

pH can be created using the Henderson–Hasselbalch equation. However, the predictions of all these equations are somewhat approximate unless the activity of acids and conjugate bases are taken into account.

Titratable acidity provides a simple estimate of the total acid content of a food. In most cases, it is only an estimate since foods often contain many acids that cannot be differentiated through titration. Titratable acidity is not a good predictor of pH, since pH is a combined function of titratable acid and conjugate base. Instrumental methods such as HPLC and electrochemical approaches measure acids and their conjugate bases as a single compound and, therefore, tend to produce acid contents that are higher than those determined by titration. Titratable acidity, somewhat curiously, is a better predictor of tartness than the concentration of free hydronium ions as reflected by pH. The perception of tartness is strongly influenced by the presence of sugars. Indicator dyes are used commonly to identify the endpoint of acidity titrations although pH meters can be used in critical work or when sample color makes indicators impractical.

7.6 STUDY QUESTIONS

1. Explain the theory of potentiometry and the Nernst equation as they relate to being able to use a pH meter to measure H^+ concentration.

2. Explain the difference between a saturated calomel electrode and a silver–silver chloride electrode; describe the construction of a glass electrode and a combination electrode.

3. You return from a two-week vacation and ask your lab technician about the pH of the apple juice sample you gave him or her before you left. Having forgotten to do it before, the technician calibrates a pH meter with one standard buffer stored next to the meter and then reads the pH of the sample of unpasteurized apple juice immediately after removing it from the refrigerator (40°C), where it has been stored for two weeks. Explain the reasons why this stated procedure could lead to inaccurate or misleading pH values.

4. For each of the food products listed below, what acid should be used to express titratable acidity?
   a. orange juice
   b. yogurt
   c. apple juice
   d. grape juice

5. What is a "Brix/acid ratio," and why is it often used as an indicator of flavor quality for certain foods, rather than simply Brix or acid alone?

6. How would you recommend determining the endpoint in the titration of tomato juice to determine the titratable acid? Why?

7. The titratable acidity was determined by titration to a phenolphthalein endpoint for a boiled and unboiled clear carbonated beverage. Which sample would you expect to have a higher calculated titratable acidity?

Why? Would you expect one of the samples to have a fading endpoint? Why?

8. Why and how is an ascarite trap used in the process of determining titratable acidity?

9. Why is volatile acidity useful as a measure of quality for acetic acid fermentation products, and how is it determined?

10. What factors make potassium acid phthalate a good choice as a standard acid for use in standardizing NaOH solutions to determine titratable acidity?

11. Could a sample that is determined to contain 1.5% acetic acid also be described as containing 1.5% citric acid? Why or why not?

12. An instructor was grading lab reports of students who had determined the titratable acidity of grape juice. One student had written that the % titratable acidity was 7.6% citric acid. Give two reasons why the answer was marked wrong. What would have been a more reasonable answer?

7.7 PRACTICE PROBLEMS

1. How would you prepare 500 ml of 0.1 M NaH_2PO_4 starting with the solid salt?

2. Starting with reagent grade sulfuric acid (36 N), how would you prepare 1 liter of 2 M H_2SO_4? How many ml of 10 N NaOH would be required to neutralize this acid?

3. How would you prepare 250 ml of 2 N HCl starting with reagent grade HCl (12 N)?

4. How would you prepare 1 liter of 0.04 M acetic acid starting with reagent grade HOAc (17 M)?

5. How would you prepare 150 ml of 10% NaOH?

6. If about 8.7 ml of saturated NaOH is required to prepare 1 liter of 0.1 N NaOH, how would you prepare 100 ml of 1 N NaOH?

7. What is the normality of a (1 + 3) HCl solution?

8. You are performing a titration on duplicate samples and duplicate blanks that require 4 ml of 1 N NaOH per titration sample. The lab has 10% NAOH and saturated NAOH. Choose one and describe how you would prepare the needed amount of NaOH solution.

9. Is a 1% HOAc solution the same as a 0.1 M solution? Show calculations.

10. Is a 10% NaOH solution the same as a 1 N solution? Show calculations.

11. What is the normality of a 40% NaOH solution?

12. You are performing duplicate titrations on five samples that require 15 ml of 6 N HCl each. How would you prepare the needed solution from reagent grade HCl?

13. What is the pH of a 0.057 M HCl solution?

14. Vinegar has a [H^+] of 1.77 × 10^{-3} M. What is the pH? What is the major acid found in vinegar, and what is its structure?

15. Orange juice has a [H^+] of 2.09 × 10^{-4} M. What is the pH? What is the major acid found in orange juice and what is its structure?

16. A sample of vanilla yogurt has a pH of 3.59. What is the [H^+]? What is major acid found in yogurt and what is its structure?

17. An apple pectin gel has a pH of 3.30. What is the [H^+]?

What is the major acid found in apples and what is its structure?

18. How would you make 100 ml of a 0.1 N solution of potassium acid phthalate (KHP)?

19. How would you make 100 ml of a citrate buffer that is 0.1 N in both citric acid (anhydrous) and potassium citrate $KH_2C_6H_5O_7$ (MW 230.22)?

20. What would be the pH of the 0.1 N citrate buffer described in Problem 19?

21. How would you make 1 liter of 0.1 N NaOH solution from an 18 N stock solution?

22. A stock base solution assumed to be 18 N was diluted to 0.1 N. KHP standardization indicated the normality of the working solution was 0.088 N. What was the actual normality of the solution?

23. A 20-ml sample of juice requires 25 ml of 0.1 N NaOH titrant. What would be the percent acid if the juice is (1) apple juice, (2) orange juice, (3) grape juice?

24. A lab analyzes a large number of orange juice samples. All juice samples will be 10 ml. It is decided that 5 ml of titrant should equal 1% citric acid. What base normality should be used?

25. A lab wishes to analyze apple juice. They would like each milliliter of titrant to equal 0.1% malic acid. Sample aliquots will all be 10 ml. What base normality should be used?

Answers

1. The question asks for 500 ml of a 0.1 M NaH_2PO_4 solution. The molecular weight of this salt is 120 g/mole. You can use Equation [1] to solve this problem.

    (500 ml of sodium phosphate) × (molarity of sodium phosphate) = millimoles of sodium phosphate

    $$\frac{(500 \text{ ml})(0.1 \text{ M})(120 \text{ g/mole})}{1000 \text{ ml/liter}} = 6 \text{ g NaH}_2\text{PO}_4$$

2. (a) 1000 ml of 2 M H_2SO_4 are required. Reagent grade H_2SO_4 is 36 N and 18 M. Therefore,

    (18 M) (x ml) = (2 M)(1000 ml)

    x ml = 111.1 ml of conc. acid diluted to 1 liter

    (When diluting concentrated acids, always add concentrated acid to about one half the final volume of water to dilute and to dissipate the heat generated by mixing. Never add the water to the concentrated acid!).

    (b) (1000 ml H_2SO_4)(2 M H_2SO_4)(2 N/1 M) = (x ml NaOH)(10 N NaOH)

    x ml = 400 ml NaOH

3. Using Equation [1]:

    (250 ml)(2 N HCl) = (x ml) (12 N HCl)

    x ml = 41.67 ml of conc. HCl diluted with water to 250 ml

4. Using Equation [1]:

    (0.04 M HOAc)(1 liter)(1000 ml/liter) = (x ml)(17 M HOAc)

    x ml = 2.35 ml conc. acetic acid which is diluted to 1 liter

5. Usually with a solid starting material like NaOH, the %

is a weight-to-volume % (or % wt/vol). Therefore, 10% NaOH = 10 g NaOH/100 ml of solution. Thus, 150 ml of 10% NaOH requires 15 g NaOH = 15 g NaOH/150 ml = 10% NaOH.

6. If about 8.7 ml of saturated NaOH diluted to 1 liter gives 0.1 N, this equals (0.1 N)(1000 ml) = 100 mEq. Since both solutions contain the same number of milliequivalents, they both must require the some volume of saturated NaOH, 8.7 ml.

7. The convention (1 + 3) HCl, as used for some analytical food methods (e.g., AOAC Methods), means 1 part concentrated acid and 3 parts distilled water, or a 1-in-4-dilution. Starting with concentrated HCl at 12 N, a 1-in-4 dilution will yield (1/4)(12 N HCl) = 3.00 N HCl.

8. Four titrations of 4 ml each will be performed requiring a total of about 16 ml of 1 N NaOH. For simplicity, 20 ml of 1 N NaOH can be prepared. If a 10% NaOH stock solution is used then:

    10 g NaOH/100 ml = 100 g NaOH/liter = 2.5 N NaOH
    (20 ml)(1 N NaOH) = (x ml) (2.5 N)
    x ml = 8 ml of 10% diluted to 20 ml with distilled water

    If saturated NaOH is used, remember from Problem 6 that approximately 8.7 ml of saturated NaOH diluted to 100 ml yields 1.0 N. Therefore, 1.87 ml or 2 ml of saturated NaOH diluted to 20 ml with distilled water will yield about 1 N NaOH.

9. 1% HOAc = 1 g HOAc/100 ml =
    10 g HOAc/1 liter/60.05 g/mole = 0.17 mole/liter = 0.17 M
    and
    0.1 M HOAc = 0.1 mole HOAc/liter × 60.05 g/mole
    6.005 g HOAc/liter = 0.60 g/100 ml = 0.60% HOAc.
    Therefore, the two acetic acid solutions are not the same, differing by a factor of about 2.

10. 10% NaOH = 10 g NaOH/100 ml = 100 g NaOH/liter

    100 g NaOH/(40 g/mole)/liter = 2.5 M NaOH

    and

    1 N NaOH = 1 mole NaOH/liter = 40 g NaOH/liter

    4 g NaOH/100 ml = 4% NaOH

    No, the solutions are not the same.

11. 40% NaOH = 40 g NaOH/100 ml = 400 g NaOH/liter
    (400 g NaOH/liter)/(40 g NaOH/mole) = 10 moles/liter
    = 10 N

12. A total of (5 samples)(2 duplicates)(15 ml) = 150 ml of 6 N HCl
    (150 ml)(6 N HCl) = (x ml)(12 N HCl)
    x ml = 75 ml concentrated HCl diluted with distilled water to 150 ml

13. Since HCl is a strong acid, it will be completely dissociated. Therefore, the molar concentration of HCl is the molar concentration of H^+ and of Cl^-.

    $(H^+) = 0.057$ N 5.7 × 10^{-2} M

    pH = $-\log$ (5.7 × 10^{-2})M
    = (0.76 − 2)
    = −(−1.24)
    = 1.24

What is the pH of a 0.025 N NaOH solution?

$(OH^-) = 0.025\ M = 2.5 \times 10^{-2}\ M$ 　　pOH = $-\log (2.5 \times 10^{-2})$

$= -(0.40 - 2)$

$= -(0.40 - 2)$

pH = $14 - 1.6$

$= 12.40$

How many grams of NaOH are required to make 100 ml of 0.5 N NaOH?

100 ml NaOH × 0.5 N = 50 mEq or 0.050 Eq

Since NaOH has molecular weight of 40.0 g/mole and one equivalent per mole, the equivalent weight is 40.0 g per equivalent.

14. 2.75; acetic acid:

$$\begin{array}{c} H \\ | \\ H-C-COOH \\ | \\ H \end{array}$$
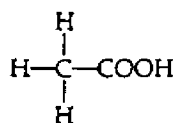
(Use the equation in Step 1 of Table 7-4, pH = $-\log$ [H$^+$], to solve Problems 14-17.)

15. 3.68; citric aid;

$$\begin{array}{ccc} COOH & COOH & COOH \\ | & | & | \\ H-C & C & C-H \\ | & | & | \\ H & OH & H \end{array}$$

16. 1.1 × 10^{-4} M; lactic acid;

$$\begin{array}{c} OH \\ | \\ CH_3-C-COOH \\ | \\ H \end{array}$$
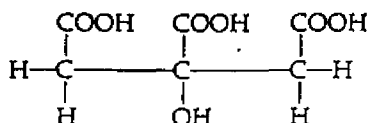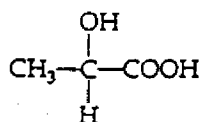
17. 5.0 × 10^{-4} M; malic acid;

$$\begin{array}{cc} COOH & COOH \\ | & | \\ HO-C & C-H \\ | & | \\ H & H \end{array}$$
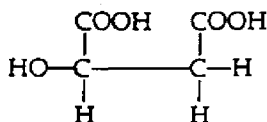
18. From Table 7-2, the equivalent weight of KHP is 204.22 g/Eq. The weight of KHP required can be calculated from the equation.

$$\text{Acid wt} = \frac{\text{Desired volume (ml)}}{1000\ \text{ml/liter}}$$

$$\times \text{Eq wt (g/Eq)}$$

$$\times \text{desired } N \text{ (Eq/liter)}$$

Therefore,

$$\text{KHP wt} = \frac{100\ \text{ml}}{1000\ \text{ml/liter}}$$

$$\times 204\ \text{g/Eq} \times 0.1\ \text{Eq/liter}$$

$$= 2.0422\ \text{g}$$

The solution can be made by weighing exactly 2.0422 g of cool, dry KHP into a 100-ml volumetric flask and diluting to volume.

19. This problem is the same as Problem 18, except two components are being added to 100 ml of solution. From Table 7-2, the equivalent weight of citric acid (anhydrous) is 64.04 g/Eq. Therefore, the weight of citric acid (CA) would be

$$\text{CA wt} = \frac{100\ \text{ml}}{1000\ \text{ml/liter}}$$

$$\times 64.04\ \text{g/Eq}$$

$$\times 0.1\ \text{Eq/liter}$$

$$= 0.6404\ \text{g}$$

Potassium citrate (PC) is citric acid with one of its three hydrogen ions removed. Consequently, it has one less equivalent per mole than CA. The equivalent weight of PC would be its molecular weight (230.22) divided by its two remaining hydrogen ions, or 115.11 g per equivalent. Therefore, the weight contribution of PC would be

$$\text{PC wt} = \frac{100\ \text{ml}}{1000\ \text{ml}}$$

$$\times 115.11\ \text{g/Eq} \times 0.1\ \text{Eq/liter}$$

$$= 1.511\ \text{g}$$

20. The relationship between pH and conjugate acid/base pair concentrations is given by the Henderson-Hasselbalch equation.

$$pH = pK_a + \log \frac{[A^-]}{[HA]}$$

When acid and conjugate base concentrations are equal, [A$^-$]/[HA] = 1. Since the log of 1 is 0, the pH will equal the pK_a of the acid. Because CA and PC are both 0.1 N, the pH will equal the pK_a1 of citric acid given in Table 7-5 (pH = 3.2).

21. Using Equation [1] and solving for volume of concentrate, we get

$$\text{ml concentrated solution} = \frac{\text{final } N \times \text{final ml}}{\text{beginning } N}$$

$$= \frac{0.1\ N \times 1000\ \text{ml}}{18\ N}$$

$$= 5.55\ \text{ml}$$

Consequently, 5.55 ml would be dispensed into a 1-liter volumetric flask. The flask would then be filled to volume with distilled CO$_2$-free water.

The normality of this solution will only be approximate since NaOH is not a primary standard. Standardization against a KHP solution or some other primary standard is essential. It is useful sometimes to back-calculate the true normality of the stock solution. Even under the best circumstances, the normality will decrease with time, but back-calculating will permit a closer approximation of the target normality the next time a working standard is prepared.

22. This answer is a simple ratio.

$$\frac{0.088}{0.100} \times 18 = 15.85\ N$$

In general chemistry, acid strength is frequently reported in normality. However, food acids are reported usually as percent of total sample weight. Trace acids also may be reported in milligram percent (mg/100 g of sample).

Titratable acidity measurements require relatively small acid and titrant volumes. Normalities for small volumes are typically reported in milliequivalents per milliliter (mEq/ml). Normalities reported in Eq/liter are numerically identical to normalities reported in mEq/ml, only the unit portions differ. Therefore, a 1 normal solution of NaOH contains 1 equivalent of NaOH per liter or 1 milliequivalent per milliliter. The percent acid can be calculated by the equation

$$\% \text{ acid} = \frac{\begin{array}{c}\text{base normality (mEq/ml)}\\ \times \text{ ml base}\\ \times \text{ Eq wt of acid (mg/Eq)}\end{array}}{\text{sample weight (mg)}} \times 100$$

It is often awkward in routine work to cite sample weights in milligrams. A modification of the previous equation allows sample weights to be reported directly in grams.

$$\% \text{ acid} = \frac{\begin{array}{c}\text{base normality (mEq/ml)}\\ \times \text{ ml base}\\ \times \text{ Eq wt of acid (mg/Eq)}\end{array}}{\text{sample weight (g)} \times 10} \times 100$$

For routine titration of single-strength juice, milliliters often can be substituted for sample weight in grams. Depending on the soluble solids content of the juice, the resulting acid values will be high by 1–6%.

23. Table 7-6 indicates the principal acids in apple, orange, and grape juice are malic, citric, and tartaric acids, respectively. Table 7-2 indicates the equivalent weight of these acids are malic (67.05), citric (64.04), and tartaric (75.05). The percent acid for each of these juices would be

$$\text{malic acid} = \frac{\begin{array}{c}0.1 \text{ mEq/ml NaOH}\\ \times 25 \text{ ml}\\ \times 67.05 \text{ mg/mEq}\end{array}}{20 \text{ ml (10) mg/ml}} = 0.84$$

$$\text{citric acid} = \frac{\begin{array}{c}0.1 \text{ mEq/ml NaOH}\\ \times 25 \text{ ml}\\ \times 64.04 \text{ mg/mEq}\end{array}}{20 \text{ ml (10) mg/ml}} = 0.80$$

$$\text{tartaric acid} = \frac{\begin{array}{c}0.1 \text{ mEq/ml NaOH}\\ \times 25 \text{ ml}\\ \times 75.05 \text{ mg/mEq}\end{array}}{20 \text{ ml (10) mg/ml}} = 0.94$$

The three values obtained for the different acids are closer than one might expect from a casual examination of molecular weights. When two acids predominate in a juice, they are usually malic and citric or malic and tartaric acids. Choosing the wrong predominant acid does not seriously affect the calculated percent acid since equivalent weights of naturally occurring food acid are similar.

Notice that the equivalent weight of anhydrous citric acid was used. The anhydrous form always will be used in calculating and reporting the results of titration. Pure citric acid has a tendency to absorb water. Some manufacturers of pure citric acid intentionally hydrate the molecule to stabilize it against further hydration. The equivalent weight of citric acid monohydrate will be used only when making solutions from hydrated starting materials. Hydrated chemicals should never be dried in a drying oven. Total dehydration is rarely possible. The resulting compound would have some intermediate (and unknown) hydration number, and solutions made from the compound would be inaccurate.

Quality control laboratories often analyze a large number of samples having a specific type of acid. Speed and accuracy are increased if acid concentration can be read directly from the burette. It is possible to adjust the normality of the base to achieve this purpose. The proper base normality can be calculated from the equation

$$N = \frac{10 \times A}{B \times C}$$

where:

A = weight (or volume) of the sample to be titrated
B = volume (ml) of titrant you want to equal 1% acid
C = equivalent weight of the acid

24.

$$N = \frac{10 \times 10}{5 \times 64.04} = 0.3123 \, N$$

In actuality, the standard alkali solution used universally by the Florida citrus industry is 0.3123 N.

25. Since each milliliter will equal 0.1% malic acid, 1% malic acid will equal 10 ml. Therefore,

$$N = \frac{10 \times 10}{5 \times 67.05} = 0.1491 \, N$$

7.8 RESOURCE MATERIALS

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ACKNOWLEDGMENT
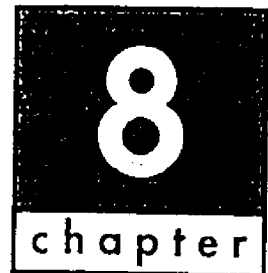
Moisture and Total Solids Analysis

Robert L. Bradley, Jr.

8.1 INTRODUCTION

Moisture assays can be one of the most important analyses performed on a food product and yet one of the most difficult from which to obtain accurate and precise data. This chapter describes various methods for moisture analysis—their principles, procedures, applications, cautions, advantages, and disadvantages. Water activity measurement also is described, since it parallels the measurement of total moisture as an important quality factor. With an understanding of techniques described, one can apply appropriate moisture analyses to a wide variety of food products.

8.1.1 Importance of Moisture Assay

One of the most fundamental and important analytical procedures that can be performed on a food product is an assay for the amount of moisture (1–3). The dry matter that remains after moisture removal is commonly referred to as total solids. This analytical value is of great economic importance to a food manufacturer because water is an inexpensive filler. The following listing gives some examples in which moisture content is important to the food processor.

1. Moisture is a quality factor in the preservation of some products and affects stability in
   a. dehydrated vegetables and fruits
   b. dried milks
   c. powdered eggs
   d. dehydrated potatoes
   e. spices and herbs
2. Moisture is used as a quality factor for
   a. jams and jellies, to prevent sugar crystallization
   b. sugar syrups
   c. prepared cereals—conventional, 4–8%; puffed, 7–8%
3. Reduced moisture is used for convenience in packaging or shipping of
   a. concentrated milks
   b. liquid cane sugar (67% solids) and liquid corn sweetener (80% solids)
   c. dehydrated products (these are difficult to package if too high in moisture)
   d. concentrated fruit juices
4. Moisture (or solids) content is often specified in compositional standards (i.e., Standards of Identity)
   a. Cheddar cheese must be ≤39% moisture.
   b. Enriched flour must be ≤15% moisture.
   c. Pineapple juice must have soluble solids of ≥10.5° Brix (conditions specified).
   d. Glucose syrup must have ≥70% total solids.

e. The percentage of added water in processed meats is commonly specified.
5. Computations of the nutritional value of foods require that you know the moisture content.
6. Moisture data are used to express results of other analytical determinations on a uniform basis (i.e., dry weight basis).

8.1.2 Moisture Content of Foods

The moisture content of foods varies greatly, as shown in Table 8-1. Water is a major constituent of most food products. The approximate, expected moisture content of a food can affect the choice of the method of measurement. It also can guide the analyst in determining the practical level of accuracy required when measuring moisture content, relative to other food constituents.

8.1.3 Forms of Water in Foods

The ease of water removal from foods depends on how it exists in the food product. The three states of water in food products are:

1. Free water—This water retains its physical properties and thus acts as the dispersing agent for colloids and the solvent for salts.
2. Adsorbed water—This water is held tightly or is occluded in cell walls or protoplasm and is held tightly to proteins.
3. Water of hydration—This water is bound chemically, for example, lactose monohydrate; also some salts such as $Na_2SO_4 \cdot 10H_2O$.

Depending on the form of the water present in a food, the method used for determining moisture may measure more or less of the water present. This is the reason for official methods with stated procedures (5–7). However, several official methods may exist for a particular product. For example, the AOAC International methods for cheese include: method 926.08, vacuum oven; 948.12, forced draft oven; 977.11, microwave oven; 969.19, distillation (5). Usually, the first method listed by AOAC International is preferred over others in any section.

8.1.4 Sample Collection and Handling

General procedures for sampling, sample handling and storage, and sample preparation are given in Chapter 5. These procedures are perhaps the greatest potential source of error in any analysis. Precautions must be taken to minimize inadvertent moisture losses or gains that occur during these steps. Obviously, any exposure of a sample to the open atmosphere should be as short as possible. Any heating of a

8-1 table	

Moisture Content of Selected Foods

Food Item	Approximate Percent Moisture (wet weight basis)
Cereals, bread, and pasta	
Wheat flour, whole-grain	10.3
White bread, enriched	13.4
Corn flakes cereal	3.0
Crackers saltines	4.1
Macaroni, dry, enriched	10.2
Dairy products	
Milk, whole, fluid, 3.3% fat	88.0
Yogurt, plain, low fat	89.0
Cottage cheese	79.3
Cheddar cheese	37.5
Ice cream, vanilla	61.0
Fats and oils	
Margarine, regular, hard, corn	16.7
Butter, with salt	16.9
Oil—soybean, salad or cooking	0
Fruits and vegetables	
Watermelon, raw	91.5
Oranges, raw	86.8
Apples, raw, with skin	83.9
Grapes, American type, raw	81.3
Raisins	15.4
Cucumbers, with peel, raw	96.0
Potatoes, raw, flesh and skin	79.0
Snap beans, green, raw	90.3
Meat, poultry, and fish	
Beef, ground, extra lean, raw	63.2
Chicken, broilers and fryers, light meat, meat and skin, raw	68.6
Finfish, flatfish (flounder and sole species), raw	79.1
Egg, whole, raw, fresh	75.3
Nuts	
Walnuts, black, dried	4.4
Peanuts, all types, dry roasted with salt	1.6
Peanut butter, smooth style, with salt	1.2
Sweeteners	
Sugar, granulated	0
Sugar, brown	1.6
Honey, strained or extracted	17.1

From USDA Nutrient Database for Standard Reference, with modification. Release 11-1 (August 1997).
http://www.nai.usda.gov/fnic/cgi-bin/nut_search.pl

sample by friction during grinding should be minimized. Headspace in the sample storage container should be minimal because moisture is lost from the sample to equilibrate the container environment against the sample.

To illustrate the need for optimum efficiency and speed in weighing samples for analysis, Vanderwarn (8) showed, using shredded Cheddar cheese (2–3 g in a 5.5-cm aluminum foil pan), that moisture loss within an analytical balance was a straight line function. The rate of loss was related to the relative humidity. At 50% relative humidity, it required only 5 sec to lose 0.01% moisture. This time doubled at 70% humidity, or 0.01% moisture loss in 10 sec. While one might expect

a curvilinear loss, the moisture loss was actually linear over a 5-min study interval. These data demonstrate the necessity of absolute control during collection of samples through weighing, before drying.

8.2 OVEN DRYING METHODS

In oven drying methods, the sample is heated under specified conditions, and the loss of weight is used to calculate the moisture content of the sample. The moisture content value obtained is highly dependent on the type of oven used, conditions within the oven, and the time and temperature of drying. Various oven methods

are AOAC approved for determining the moisture in many food products. The methods are simple, and many ovens allow for simultaneous analysis of large numbers of samples. The time required may be from a few minutes to over 24 hr.

8.2.1 General Information

8.2.1.1 Removal of Moisture

Any oven method used to evaporate moisture has as its foundation the fact that the boiling point of water is 100°C; however, this considers only pure water at sea level. Free water is the easiest of the three forms of water to remove. However, if 1 gram molecular weight (1 mole) of a solute is dissolved in 1.0 liter of water, the boiling point would be raised by 0.512°C. This boiling point elevation continues throughout the moisture removal process as more and more concentration occurs.

Moisture removal is sometimes best achieved in a two-stage process. Liquid products (e.g., juices, milk) are commonly predried over a steam bath before drying in an oven. Products such as bread and field-dried grain are often air dried, then ground and oven dried, with the moisture content calculated from moisture loss at both air and oven drying steps. Particle size, particle size distribution, sample sizes, and surface area during drying influence the rate and efficiency of moisture removal.

8.2.1.2 Decomposition of Other Food Constituents

Moisture loss from a sample during analysis is a function of time and temperature. Decomposition enters the picture when time is extended too much or temperature is too high. Thus, most methods for food moisture analysis involve a compromise between time and a particular temperature at which limited decomposition might be a factor. One major problem exists in that the physical process must separate all the moisture without decomposing any of the constituents that could release water. For example, carbohydrates decompose at 100°C according to the following reaction:

$$C_6H_{12}O \rightarrow 6C + 6H_2O \qquad [1]$$
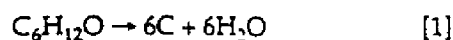
The water generated in carbohydrate decomposition is not the moisture that we want to measure. Certain other chemical reactions (e.g., sucrose hydrolysis) can result in utilization of moisture, which would reduce the moisture for measurement. A less serious problem, but one that would be a consistent error, is the loss of volatile constituents, such as acetic, propionic, and butyric acids; and alcohols, esters, and aldehydes among flavor compounds. While weight changes in oven drying methods are assumed to be due to moisture loss, weight gains also can occur due to oxidation of unsaturated fatty acids and certain other compounds.

Nelson and Hulett (9) determined that moisture was retained in biological products to at least 365°C, which is coincidentally the critical temperature for water. Their data indicate that among the decomposition products at elevated temperatures were CO, CO_2, CH_4, and H_2O. These were not given off at any one particular temperature but at all temperatures and at different rates at the respective temperature in question.

By plotting moisture liberated against temperature, curves were obtained that show the amount of moisture liberated at each temperature (Fig. 8-1). Distinct breaks were shown that indicated the temperature at which decomposition became measurable. None of these curves showed any break before 184°C. Generally, proteins decompose at temperatures somewhat lower than those required for starches and celluloses. Extrapolation of the flat portion of each curve to 250°C gave a true moisture content based on the assumption that there was no adsorbed water present at the temperature in question.

8.2.1.3 Temperature Control

Drying methods utilize specified drying temperatures and times, which must be carefully controlled. Moreover, there may be considerable variability of temperature, depending on the type of oven used for moisture analysis. One should determine the extent of variation within an oven before relying on data collected from its use.

Consider the temperature variation in three types of ovens: convection (atmospheric), forced draft, and vacuum. The greatest temperature variation exists in a convection oven. This is because hot air slowly circulates without the aid of a fan. Air movement is obstructed further by pans placed in the oven. When the oven door is closed, the rate of temperature recovery is generally slow. This is dependent also upon the load placed in the oven and upon the ambient temperature. A 10°C temperature differential across a convection oven is not unusual. This must be considered in view of anticipated analytical accuracy and precision. A convection oven should not be used when precise and accurate measurements are needed. Forced draft ovens have the least temperature differential across the interior of all ovens, usually not greater than 1°C. Air is circulated by a fan that forces air movement throughout the oven cavity. Forced draft ovens with air distribution manifolds appear to have added benefit where air movement is horizontal across shelving. Thus, no
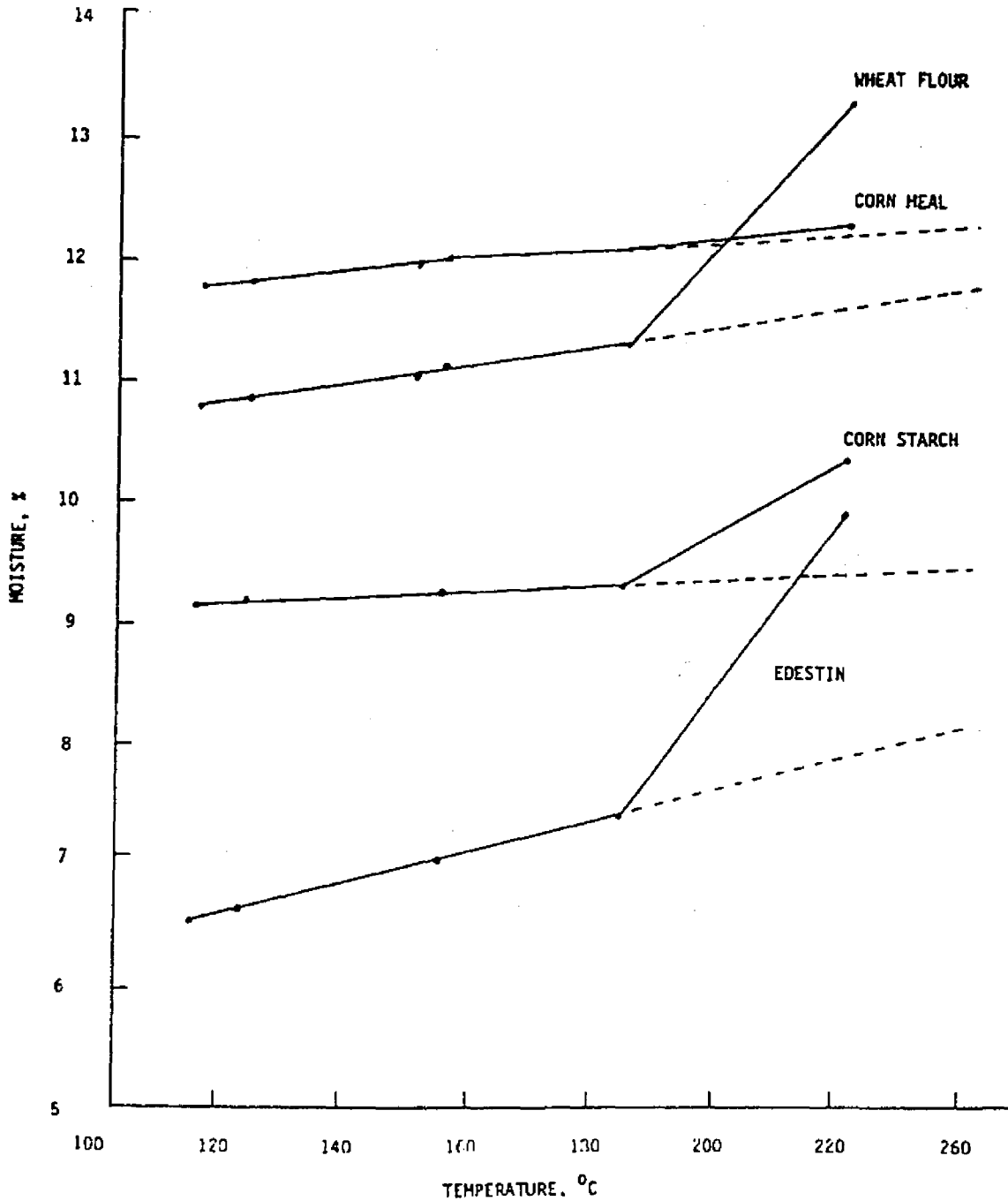
8-1
figure

Moisture content of several foods held at various temperatures in an oven. The hyphenated line extrapolates data to 250°F, the true moisture content. [Reprinted with permission from (9) Nelson, O.A., and Hulett, G.A. 1920. The moisture content of cereals. *Journal of Industrial and Engineering Chemistry* 12:40–45. Copyright 1920, American Chemical Society.]

matter whether the oven is filled with moisture pans or only half filled, the result would be the same for a particular sample. This has been demonstrated using a Lab-Line oven (Melrose Park, IL) in which three stacking configurations for the pans were used (8). In one configuration, the oven was filled with as many pans holding 2–3 g of Cheddar cheese as the forced draft

oven could hold. In the two others, one half of the full load of pans with cheese was used with the pans (1) in orderly vertical rows with the width of one pan between rows, or (2) staggered such that pans on every other shelf were in vertical alignment. The results after drying showed no difference in the mean value or the standard deviation.

Two features of some vacuum ovens contribute to a wider temperature spread across the oven. One feature is a glass panel in the door. Although from an educational point of view it may be fascinating to observe some samples in the drying state, the glass is a heat sink. The second feature is the way by which air is bled into the oven. If the air inlet and discharge are on opposite sides, conduct of air is virtually straight across the oven. Some newer models (Lab-Line model 3623) have air inlet and discharge manifolds mounted top and bottom. Air movement in this style of vacuum oven is upward from the front, then backward to the discharge in a broad sweep. The effect is to minimize cold spots as well as to exhaust moisture in the interior air.

8.2.1.4 Types of Pans for Oven Drying Methods

Pans used for moisture determinations are varied in shape and may or may not have a cover. The AOAC International (5) moisture pan is about 5.5 cm in diameter with an insert cover. Other pans have covers that slip over the outside edge of the pan. These pans, while reusable, are expensive, particularly in terms of labor costs to clean appropriately to allow reuse.

Pan covers are necessary to control loss of sample by spattering during the heating process. If the cover is metal, it must be slipped to one side during drying to allow for moisture evaporation. However, this slipping of the cover also creates an area where spattering will result in product loss. Examine the interior of most moisture ovens and you will detect odor and deposits of burned-on residue, which, although undetected at the time of occurrence, produce erroneous results and large standard deviations (8).

Consider the use of disposable pans whenever possible; then purchase glass fiber discs for covers. At 5.5 cm in diameter, these covers fit perfectly inside disposable aluminum foil pans and prevent spattering while allowing the surface to breathe. Paper filter discs foul with fat and thus do not breathe effectively. Consider the evidence presented in Fig. 8-2, derived from at least 10 replicate analyses of the same cheese with various pans and covers. These data prove two points: (1) fat does spatter from pans with slipped covers and (2) fiberglass is the most satisfactory cover.

8.2.1.5 Handling and Preparation of Pans

The handling and preparation of pans before use requires consideration. Use only tongs to handle any pan. Even fingerprints have weight. All pans must be oven treated to prepare them for use. This is a factor of major importance unless disproved by the technologist doing moisture determinations with a particular type of pan. To illustrate the weight change that occurs with disposable aluminum pans, consider the examples in Fig. 8-3. Disposable aluminum pans must be vacuum oven dried for 3 hr before use. At 3 hr and 15 hr in either a vacuum or forced draft oven at 100°C, pans varied in their weight within the error of the balance, or 0.0001 g (8). Store dry moisture pans in a functioning desiccator. The glass fiber covers do not need drying before use.

8.2.1.6 Control of Surface Crust Formation (Sand Pan Technique)

Some food materials tend to form a semipermeable crust or to lump together during drying, which will contribute to erratic and erroneous results. To control this problem, analysts use the sand pan technique. Clean, dry sand and a short glass stirring rod are preweighed into a moisture pan. Subsequently, after weighing in a sample, the sand and sample are admixed with the stirring rod left in the pan. The remainder of the procedure follows a standardized method if available; otherwise the sample is dried to constant weight. The purpose of the sand is twofold: to prevent surface crust from forming and to disperse the sample so evaporation of moisture is less impeded. The amount of sand used is a function of sample size. Consider 20–30 g sand/3 g sample to obtain desired distribution in the pan. Similar to the procedure, applications, and advantages of using sand, other heat-stable inert materials such as diatomaceous earth can be used in moisture determinations, especially for sticky fruits. These inert matrices function to disperse the food constituents and minimize the retention of moisture in the food products.

8.2.1.7 Calculations

Moisture and total solids contents of foods can be calculated as follows using oven drying procedures:

$$\% \text{ Moisture (wt/wt)} = \frac{\text{wt } H_2O \text{ in sample}}{\text{wt of wet sample}} \times 100 \quad [2]$$

$$\% \text{ Moisture (wt/wt)} = \frac{\text{wt of wet sample} - \text{wt of dry sample}}{\text{wt of wet sample}} \times 100 \quad [3]$$

$$\% \text{ Total solids (wt/wt)} = \frac{\text{wt of dry sample}}{\text{wt ot wet sample}} \times 100 \quad [4]$$

8.2.2 Forced Draft Oven

When using a forced draft oven, the sample is rapidly weighed into a moisture pan and placed in the oven for
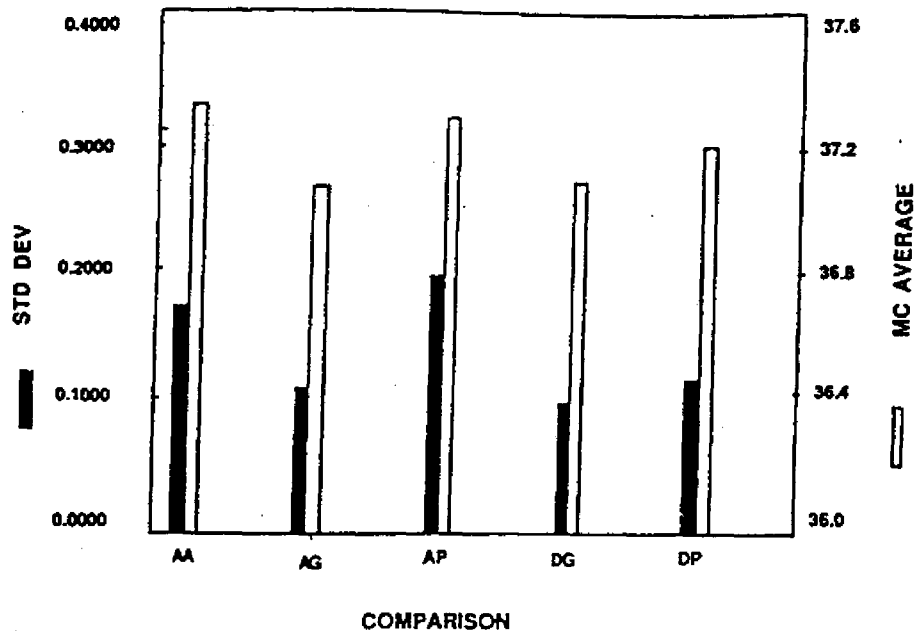
8-2
figure

Effect of various pan and cover combinations on the moisture content (MC) of Cheddar cheese. Standard deviations show precision of the analysis. Pans: A = AOAC, D = disposable; Covers: A = AOAC, G = glass fiber disc, P = filter paper disc. [From (8), used with permission.]

an arbitrarily selected time if no standardized method exists. Drying time periods for this method are 0.75–24 hr (Table 8-2), depending on the food sample and its pretreatment; some liquid samples are dried initially on a steam bath at 100°C to minimize spattering. In these cases, drying times are shortened to 0.75–3 hr. A forced draft oven is used with or without a steam table predrying treatment to determine the solids content of fluid milks (AOAC Method 990.19, 990.20).

An alternative to selecting a time period for drying is to weigh and reweigh the dried sample and pan until two successive weighings taken 30 min apart agree within a specified limit, for example, 0.1–0.2 mg for a 5-g sample. The user of this second method must be aware of sample transformation, such as browning, which suggests moisture loss of the wrong form. Samples high in carbohydrates should not be dried in a forced draft oven but rather in a vacuum oven at a temperature no higher than 70°C. Lipid oxidation and a resulting sample weight gain can occur at high temperatures in a forced draft oven.

8.2.3 Vacuum Oven

By drying under reduced pressure (25–100 mm Hg), one is able to obtain a more complete removal of water and volatiles without decomposition within a 3–6 hr drying time. Vacuum ovens need a dry air purge in addition to temperature and vacuum controls to oper-

ate within method definition. In older methods, a vacuum flask is used, partially filled with concentrated sulfuric acid as the desiccant. One or two air bubbles per second are passed through the acid. Recent changes now stipulate an air trap that is filled with calcium sulfate containing an indicator to show moisture saturation. Between the trap and the vacuum oven is an appropriately sized rotameter to measure air flow (100–120 ml/min) into the oven.

The following are important points in the use of a vacuum drying oven:

1. Temperature used depends on the product, such as 70°C for fruits and other high-sugar products. Even with reduced temperature, there can be some decomposition.
2. If the product to be assayed has a high concentration of volatiles, you should consider the use of a correction factor to compensate for the loss.
3. Analysts should remember that in a vacuum, heat is not conducted well. Thus pans must be placed directly on the metal shelves to conduct heat.
4. Evaporation is an endothermic process; thus, a pronounced cooling is observed. Because of the cooling effect of evaporation, when several samples are placed in an oven of this type, you will note that the temperature will drop. Do not attempt to compensate for the cooling effect by
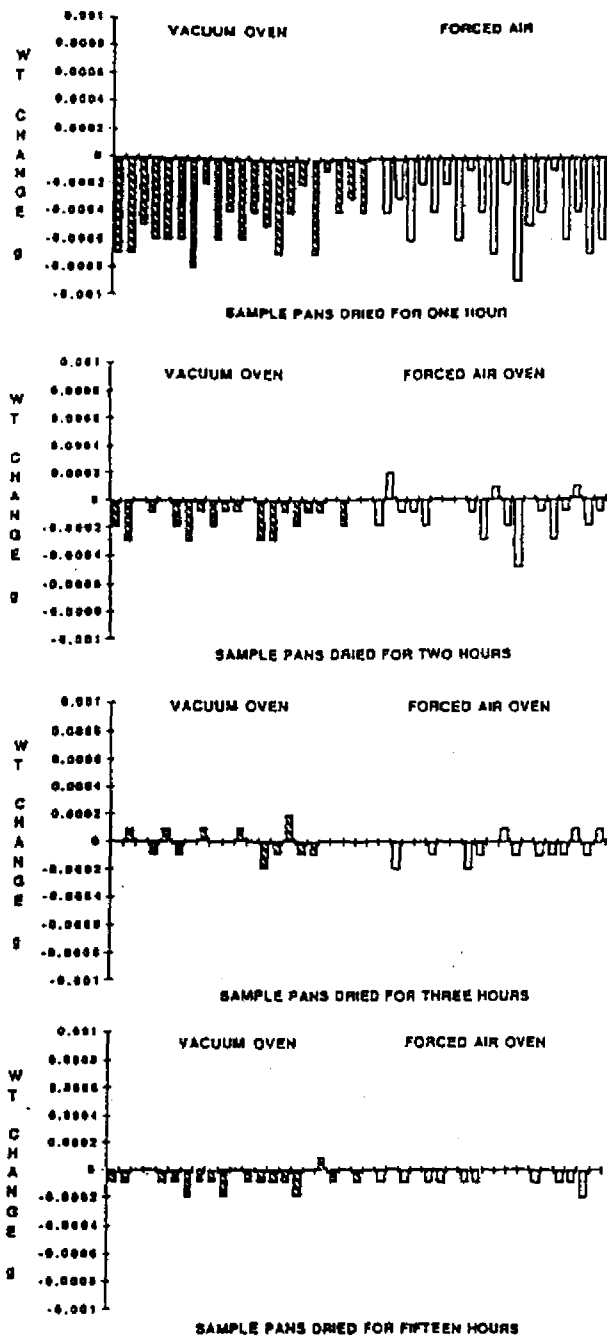
8-3
figure

Effect of drying new disposable aluminum moisture pans in a vacuum and forced draft oven at 100°C. The sensitivity of the balance was 0.0001 g. [From (8), used with permission.]

increasing the temperature; otherwise samples during the last stages of drying will be overheated.

5. The drying time is a function of the total moisture present, nature of the food, surface area per unit weight of sample, whether sand is used as

a dispersant, and the relative concentration of sugars and other substances capable of retaining moisture or decomposing. The drying interval is determined experimentally to give reproducible results.

8.2.4 Microwave Oven

Microwave oven drying in its infancy was looked upon as a great boon to moisture determination. It was the first precise and rapid technique that allowed some segments of the food industry to make in-process adjustment of moisture in the food before final packaging. For example, process cheese could be analyzed and the composition adjusted before the blend was dumped from the cooker. Such control could effectively pay for the microwave oven within a few months. Methods texts indicated that users must check results against the AOAC vacuum oven procedure to determine how much microwave energy was needed. Adjustment of megatron output on the original Apollo oven was done by turning a control knob.

A new model from CEM Corporation (Matthews, NC) had significant improvements over the original Apollo oven. A particular microwave oven, or equivalent, is specified in the AOAC International procedures for moisture analysis of cheese (AOAC Method 977.11), total solids analysis of processed tomato products (AOAC Method 985.26), and moisture analysis of meat and poultry products (AOAC Method 985.14).

With the CEM microwave oven, the user controls the microwave output by setting the microprocessor controller to a percentage of full power. Next the internal balance is tared with two fiberglass pads on the balance. As rapidly as possible, a sample is placed between the two pads and weighed against the tare weight. Time for the drying operation is set by the operator and "start" is activated. The microprocessor controls the drying procedure, with percentage moisture indicated in the controller window.

The procedure described above suffers somewhat from a few inherent difficulties. The focus of the microwave energy is such that unless the sample is centrally located and evenly distributed, some portions may burn while other areas are underprocessed. The amount of time needed for an inexperienced operator to place an appropriate sample weight between the pads results in too much moisture loss before weighing. Newer models may eliminate these problems.

Another style of microwave oven sold in Europe is beginning to appear in some food plants in the United States. This is a vacuum microwave oven. It will accommodate one sample in triplicate or three different samples at one time. In 10 min, the results are

| 8-2 | Forced Draft Oven Temperature and Times for Selected Foods |

Product	Dry on Steam Bath	Oven Temperature (°C ± 2)	Time in Oven (Hr)
Buttermilk, liquid	X[1]	100	3
Cheese, natural type only		100	16.5 ± 1.5
Chocolate and cocoa		100	3
Cottage cheese		100	3
Cream, liquid and frozen	X	100	3
Egg albumin, liquid	X	130	0.75
Egg albumin, dried	X	100	0.75
Ice cream and frozen desserts	X	100	3.5
Milk			
whole, low fat, and skim	X	100	3
condensed skim		100	3
evaporated		100	3
Nuts: almonds, peanuts, walnuts		130	3

From (6) p. 492, with permission, *Standard Methods for the Examination of Dairy Products*, Robert T. Marshall (Ed.) Copyright 1992 by the American Public Health Association.
[1]X = samples must be partially dried on steam bath before being placed in oven.

reported to be similar to 5 hr in a vacuum oven at 100°C. Potentially this also could become the workhorse like other microwave ovens.

A consensus is that microwave drying is sufficiently accurate to be used for routine determinations of food moisture content. Obviously the distinct advantage of rapid analysis far out weighs its limitations for accuracy with single samples (10).

8.2.5 Infrared Drying

Infrared drying involves penetration of heat into the sample being dried, as compared to heat conductivity and convection with conventional ovens. Such heat penetration to evaporate moisture from the sample can significantly shorten the required drying time, to 10–25 min. The infrared lamp used to supply heat to the sample results in a filament temperature of 2000–2500 K. Factors that must be controlled include distance of the infrared source from the dried material and thickness of the sample. The analyst must be careful that the sample does not burn or case harden while drying. Infrared drying ovens may be equipped with forced ventilation to remove moisture air and an analytical balance to read moisture content directly. No infrared drying moisture analysis techniques are approved by AOAC currently. However, because of the speed of analysis, this technique is suited for qualitative in-process use.

8.2.6 Rapid Moisture Analyzer Technology

Many rapid moisture/solids analyzers are available to the food industry. In addition to those based on infrared

and microwave drying as described previously, compact instruments that depends on high heat are available, such as analyzers that detect moisture levels from 50 ppm to 100% using sample weights of 150 mg to 40 g (e.g., Computrac, Arizona Instrument Corporation, Phoenix, AZ). Using a digital balance, the test sample is placed on an aluminum pan or filter paper and the heat control program (with a heating range of 25°C to 275°C) elevates the test sample to a constant temperature. As the moisture is driven from the sample, the instrument automatically weighs and calculates the percentage moisture or solids. This technology is utilized to cover a wide range or applications within the food industry and offers quick and accurate results within minutes. These analyzers are utilized for both production and laboratory use with results comparable to reference methods.

8.3 DISTILLATION PROCEDURES

8.3.1 Overview

Distillation techniques involve codistilling the moisture in a food sample with a high boiling point solvent that is immiscible in water, collecting the mixture that distills off, and then measuring the volume of water. Two distillation procedures are in use today: direct and reflux distillations, with a variety of solvents. For example, in direct distillation with immiscible solvents of higher boiling point than water, the sample is heated in mineral oil or liquid with a flash point well above the boiling point for water. Other immiscible liquids with boiling point only slightly above water can be used

(e.g., toluene, xylene, and benzene). However, reflux distillation with the immiscible solvent toluene is the most widely used method.

Distillation techniques were originally developed as rapid methods for quality control work, but they are not adaptable to routine testing. The distillation method is an AOAC-approved technique for moisture analysis of spices (AOAC Method 986.21), cheese (AOAC Method 969.19), and animal feeds (AOAC Method 925.04). It also can give good accuracy and precision for nuts, oils, soaps, and waxes.

Distillation methods cause less thermal decomposition of some foods than over drying at high temperatures. Adverse chemical reactions are not eliminated but can be minimized by using a solvent with a lower boiling point. This, however, will increase distillation times. Water is measured directly in the distillation procedure (rather than by weight loss), but reading the volume of water in a receiving tube may be less accurate than using a weight measurement.

8.3.2 Reflux Distillation with Immiscible Solvent

Reflux distillation uses either a solvent less dense than water (e.g., toluene, with a boiling point of 110.6°C; or xylene, with a boiling range of 137–140°C) or a solvent more dense than water (e.g., tetrachlorethylene, with a boiling point of 121°C). The advantage of using this last solvent is that material to be dried floats; therefore it will not char or burn. In addition, there is no fire hazard with this solvent.
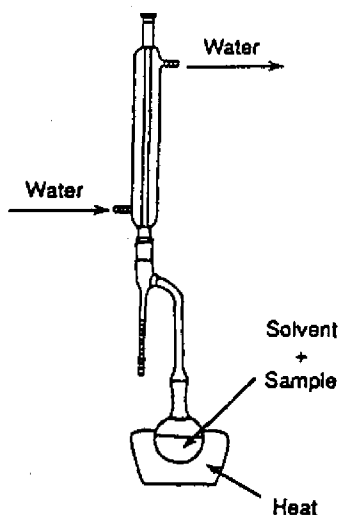
8-4 **figure** Apparatus for reflux distillation of moisture from a food. Key to this setup is the Bidwell–Sterling moisture trap. This style can be used only where the solvent is less dense than water.

A Bidwell–Sterling moisture trap (Fig. 8-4) is commonly used as part of the apparatus for reflux distillation with a solvent less dense than water. The distillation procedure using such a trap is described in Fig. 8-5, with emphasis placed on dislodging adhering water drops, thereby minimizing error. When the toluene in the distillation just starts to boil, the analyst will observe a hazy cloud rising in the distillation flask. This is a vaporous emulsion of water in toluene. As the vapors rise and heat the vessel, the Bidwell–Sterling trap, and the bottom of the condenser, condensation occurs. It also is hazy at the cold surface of the condenser, where water droplets are visible. The emulsion inverts and becomes toluene dispersed in water. This turbidity clears very slowly on cooling.

Three potential sources of error with distillation should be eliminated if observed:

1. Formation of emulsions that will not break. Usually this can be controlled by allowing the

REFLUX DISTILLATION

Place sample in distillation flask and cover completely with solvent.

⇩

Fill the receiving tube (e.g., Bidwell–Sterling Trap) with solvent, by pouring it through the top of the condenser.

⇩

Bring to a boil and distill slowly at first then at increased rate.

⇩

After the distillation has proceeded for approximately 1 hr, use an adapted buret brush to dislodge moisture droplets from the condenser and top part of the Bidwell–Sterling trap.

⇩

Slide the brush up the condenser to a point above the vapor condensing area.

⇩

Rinse the brush and wire with a small amount of toluene to dislodge adhering water drops.

⇩

If water has adhered to the walls of the calibrated turbe, invert the brush, and use the straight wire to dislodge this water so it collects in the bottom of the tube.

⇩

Return the wire to a point above the condensation point, and rinse with another small amount of toluene.

⇩

After no more water has distilled from the sample, repeat the brush and wire routine to dislodge adhering water droplets.

⇩

Rinse the brush and wire with toluene before removing from the condenser.

⇩

Allow the apparatus to cool to ambient temperatures before measuring the volume of water in the trap.

⇩

Volume of water × 2 (for a 50-g sample) = % moisture

8-5 **figure** Procedures for reflux distillation with toluene using a Bidwell–Sterling trap. Steps to dislodge adhering moisture drops are given.

apparatus to cool after distillation is completed and before reading the amount of moisture in the trap.

2. Clinging of water droplets to dirty apparatus. Clean glassware is essential, but water seems to cling even with the best cleaning effort. A burette brush, with the handle end flattened so it will pass down the condenser, is needed to dislodge moisture droplets.

3. Decomposition of the sample with production of water. This is principally due to carbohydrate decomposition to generate water ($C_6H_{12}O_6 \rightarrow 6H_2O + 6C$). If this is a measurable problem, discontinue method use and find an alternative procedure.

8.4 CHEMICAL METHOD—KARL FISCHER TITRATION

The Karl Fischer titration is particularly adaptable to food products that show erratic results when heated or submitted to a vacuum. This is the method of choice for determination of water in many low-moisture foods such as dried fruits and vegetables (AOAC Method 967.19 E-G), candies, chocolate (AOAC Method 977.10), roasted coffee, oils and fats (AOAC Method 984.20), or any low-moisture food high in sugar or protein. The method is quite rapid and sensitive and uses no heat. This method is based on the fundamental reaction described by Bunsen in 1853 (11) involving the reduction of iodine by SO_2 in the presence of water:

$$2H_2O + SO_2 + I_2 \rightarrow C_5 H_2SO_4 + 2HI \qquad [5]$$
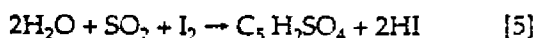
This was modified to include methanol and pyridine in a four-component system to dissolve the iodine and SO_2:

$$C_5H_5N \cdot I_2 + C_5H_5N \cdot SO_2 + C_5H_5N + H_2O$$
$$\rightarrow 2C_5H_5N \cdot HI + C_5H_5N \cdot SO_3 \qquad [6]$$

$$C_5H_5N \cdot SO_3 + CH_3OH$$
$$\rightarrow C_5H_5N(H)SO_4 \cdot CH_3 \qquad [7]$$
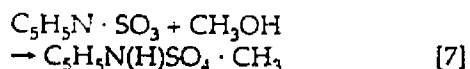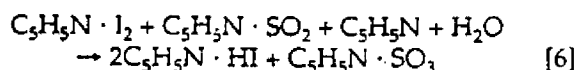
These reactions show that for each mole of water, 1 mole of iodine, 1 mole of SO_2, 3 moles of pyridine, and 1 mole of methanol are used. For general work, a methanolic solution is used that contains these components in the ratio of 1 iodine: 3 SO_2:10 pyridine, and at a concentration so that 3.5 mg of water = 1 ml of reagent. A procedure for standardizing this reagent is given below.

In a volumetric titration procedure (Fig. 8-6), iodine and SO_2 in the appropriate form are added to the sample in a closed chamber protected from atmospheric moisture. The excess of I_2 that cannot react with the water can be determined visually. The endpoint
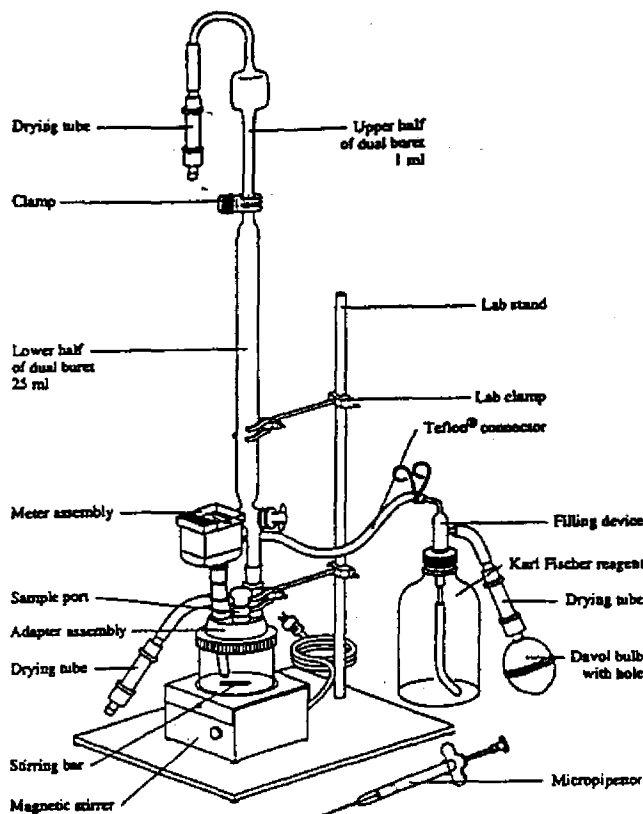
Karl Fischer titration unit. (Courtesy of Lab industries, Inc., Berkeley, CA.)

color is dark red-brown. Some instrumental systems are improved by the inclusion of a potentiometer to electronically determine the endpoint, which increases the sensitivity. Instruments are available to automatically perform the Karl Fischer moisture analysis by the conductometric method.

The volumetric titration procedure described above is appropriate for samples with a moisture content greater than ~0.03%. A second type of titration, referred to as coulometric titration, is ideal for products with very low levels of moisture, from 0.03% down to parts per million (ppm) levels. In this method, iodine is electrolytically generated to titrate the water. The amount of iodine required to titrate the water is determined by the current needed to generate the iodine.

In a Karl Fischer volumetric titration, the Karl Fischer reagent (KFR) is added directly as the titrant if the moisture in the sample is accessible. However, if moisture in a solid sample is inaccessible to the reagent, the moisture is extracted from the food with an appropriate solvent (e.g., methanol). (Particle size affects efficiency of extraction directly). Then the methanol extract is titrated with KFR.

The obnoxious odor of pyridine makes it an unde-

sirable reagent. Therefore, researchers have experimented with other amines capable of dissolving iodine and sulfur dioxide. Some aliphatic amines and several other heterocyclic compounds were found suitable. On the basis of these new amines, one-component reagents (solvent and titrant components together) and two-component reagents (solvent and titrant components separate) have been prepared. The one-component reagent may be more convenient to use, but the two-component reagent has greater storage stability.

Before the amount of water found in a food sample can be determined, a KFR water equivalence (KFReq) must be determined. The KFReq value represents the equivalent amount of water that reacts with 1 ml of KFR. Standardization must be checked before each use because the KFReq will change with time.

The KFReq can be established with pure water, a water-in-methanol standard, or sodium tartrate dihydrate. Pure water is a difficult standard to use because of inaccuracy in measuring the small amounts required. The water-in-methanol standard is premixed by the manufacturer and generally contains 1 mg of water/ml of solution. This standard can change over prolonged storage periods by absorbing atmospheric moisture. Sodium tartrate dehydrate ($Na_2C_4H_4O_6 \cdot 2H_2O$) is a primary standard for determining KFReq. This compound is very stable, contains 15.66% water under all conditions expected in the laboratory, and is the material of choice to use.

The KFReq is calculated as follows using sodium tartrate dehydrate:

$$KFReq \ (mg \ H_2O/ml) = \frac{\frac{36 \ g \ H_2O/mole}{Na_2C_4H_4O_6 \cdot 2H_2O} \times S \times 1000}{230.08 \ g/mole \times A} \quad [8]$$

where:

KFReq = Karl Fischer Reagent water equivalence
S = weight of sodium tartrate dihydrate (g)
A = ml of KFR required for titration of sodium tartrate dihydrate

Once the KFReq is known, the moisture content of the sample is determined as follows:

$$\%H_2O = \frac{KFReq \times Ks}{S} \times 100 \quad [9]$$

where:

KFReq = Karl Fischer Reagent water equivalence
Ks = ml of KFR used to titrate sample
S = weight of sample (mg)

The major difficulties and sources of error in the Karl Fischer titration methods are:

1. Incomplete water extraction—For this reason,

fineness of grind (i.e., particle size) is important in preparation of cereal grains and some foods.

2. Atmospheric moisture—External air must not be allowed to infiltrate the reaction chamber.

3. Moisture adhering to walls of unit—All glassware and utensils must be carefully dried.

4. Interferences from certain food constituents—Ascorbic acid is oxidized by KFR to dehydroascorbic acid to overestimate moisture content; carbonyl compounds react with methanol to form acetals and release water, to overestimate moisture content (this reaction also may result in fading endpoints); unsaturated fatty acids will react with iodine, so moisture content will be overestimated.

8.5 PHYSICAL METHODS

8.5.1 Electrical Methods

8.5.1.1 Dielectric Method

Moisture content of certain foods can be determined by measuring the change in capacitance or resistance to an electric current passed through a sample. These instruments require calibration against samples of known moisture content as determined by standard methods. Sample density or weight/volume relationships and sample temperature are important factors to control in making reliable and repeatable measurements by dielectric methods. These techniques can be very useful for process control measurement applications, where continuous measurement is required. These methods are limited to food systems that contain no more than 30–35% moisture.

The moisture determination in dielectric-type meters is based on the fact that the dielectric constant of water (80.37 at 20°C) is higher than that of most solvents. The dielectric constant is measured as an index of capacitance. As an example, the dielectric method is used widely for cereal grains. Its use is based on the fact that water has a dielectric constant of 80.37, whereas starches and proteins found in cereals have dielectric constants of 10. By determining this properly on samples in standard metal condensers, dial readings may be obtained and the percentage of moisture determined from a previously constructed standard curve for a particular cereal grain.

8.5.1.2 Conductivity Method

The conductivity method functions because the conductivity of an electric current increases with the percentage of moisture in the sample. A modestly accurate and rapid method is created when one measures resistance. Ohm's law states that the strength of an electricity current is equal to the electromotive force divided

by the resistance. The electrical resistance of wheat with 13% moisture is seven times as great as that with 14% moisture and 50 times that with 15% moisture. Temperature must be kept constant, and 1 min is necessary for a single determination.

8.5.2 Hydrometry

Hydrometry is the science of measuring specific gravity or density, which can be done using several different principles and instruments. While hydrometry is considered archaic in some analytical circles, it is still widely used and, with proper technique, is highly accurate. Specific gravity measurements with a pycnometer, various types of hydrometers, or a Westphal balance are commonly used for routine testing of moisture (or solids) content of numerous food products. These include beverages, salt brines, and sugar solutions. Specific gravity measurements are best applied to the analysis of solutions consisting of only one component in a medium of water.

8.5.2.1 Pycnometer

One approach to measuring specific gravity is a comparison of the weights of equal volumes of a liquid and water in standardized glassware, a pycnometer (Fig. 8-7). This will yield density of the liquid compared to water. In some texts and reference books, 20/20 is given after the specific gravity number. This indicates that the temperature of both fluids was 20°C when the weights were measured. Using a clean, dry pycnometer at 20°C, the analyst weighs it empty, fills it to the full point with distilled water at 20°C, inserts the thermometer to seal the fill opening, and then touches off the last drops of water and puts on the cap for the overflow tube. The pycnometer is wiped dry in case of any spillage from filling and is reweighed. The density of the sample is calculated as follows:

$$\frac{\text{weight of sample-filled pycnometer} - \text{weight of empty pycnometer}}{\text{weight of water-filled pycnometer} - \text{weight of empty pycnometer}} = \text{density of sample} \quad [10]$$

This method is used for determining alcohol content in alcoholic beverages (e.g., distilled liquor, AOAC Method 930.17), solids in sugar syrups (AOAC Method 932.14B), and solids in milk (AOAC Method 925.22).

8.5.2.2 Hydrometer

A second approach to measuring specific gravity is based on Archimedes' principle, which states that a
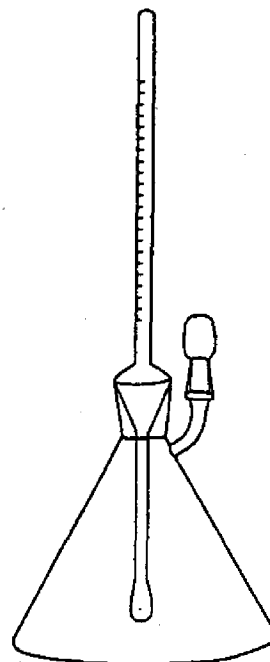
8-7
figure

Pycnometer.

solid suspended in a liquid will be buoyed by a force equal to the weight of the liquid displaced. The weight per unit volume of a liquid is determined by measuring the volume displaced by an object of standard weight. A hydrometer is a standard weight on the end of a spindle, and it displaces a weight of liquid equal to its own weight (Fig. 8-8). For example, in a liquid of low density, the hydrometer will sink to a greater depth, whereas in a liquid of high density, the hydrometer will not sink as far. Hydrometers are available in narrow and wide ranges of specific gravity. The spindle of the hydrometer is calibrated to read specific gravity directly at 15.5°C or 20°C. A hydrometer is not as accurate as a pycnometer, but the speed with which you can do an analysis is a decisive factor. The accuracy of specific gravity measurements can be improved by using a hydrometer calibrated in the desired range of specific gravities.

The rudimentary but surprisingly accurate hydrometer comes equipped with various modifications depending on the fluid to be measured:

1. The Quevenne and New York Board of Health lactometer is used to determine the density of milk. The Quevenne lactometer reads from 15 to 40 lactometer units and corresponds to 1.015 to 1.040 specific gravity. For every degree above
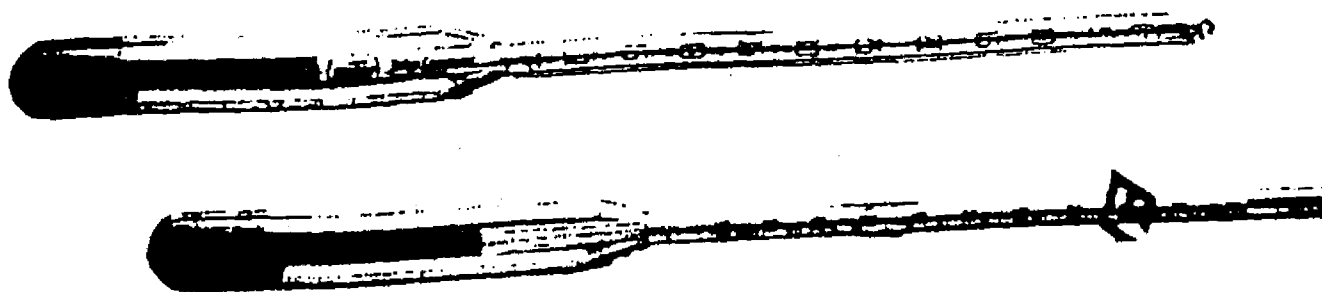
Hydrometers. (Courtesy of Cole-Parmer Instrument Company, Vernon Hills, IL.)

60°F, 0.1 lactometer units is added to the reading, and 0.1 lactometer units is subtracted for every degree below 60°F.
2. The Baumé hydrometers was used originally to determine the density of salt solutions (originally 10% salt), but it has come into much wider use. From the value obtained in the Baumé scale, you can convert to specific gravity of liquids heavier than water. For example, it is used to determine the specific gravity of milk being condensed in a vacuum pan.
3. The Brix hydrometer is a type of saccarometer used for sugar solutions such as fruit juices and syrups, and one usually reads directly the percentage of sucrose at 20°C. Balling saccharometers are graduated to indicate percentage of sugar by weight at 60°F. The terms Brix and Balling are interpreted as the weight percent of pure sucrose.
4. Alcoholometers are used to estimate the alcohol content of beverages. Such hydrometers are calibrated in 0.1 or 0.2° proof to determine percentage of alcohol in distilled liquors (AOAC Method 957.03).
5. The Twaddell hydrometer is only for liquids heavier than water.

8.5.2.3 Westphal Balance

The Westphal balance functions on Archimedes' principle such that the plummet on the balance will be buoyed by the weight of liquid equal to the volume displaced. This is more accurate than a hydrometer but less accurate than a pycnometer. It provides measurements to four decimal places. The balance has a plummet that displaces exactly 5 g of water at 15.5°C. If the specific gravity is 1, as would be the case with water at 15.5°C, a gravity weight hung at the 10 mark would bring this device into balance.

The specific gravity measurement of solid objects

is made as described below, with the determination of frozen pea maturity given as the example:

1. Weigh peas in air.
2. Immerse peas in solvent.
3. Obtain weight in this solvent.

$$\text{Specific gravity} = \frac{\text{weight in air} \times \text{specific gravity of liquid}}{\text{weight in liquid} - \text{weight in air}} \quad [11]$$

The difference between the weight in air and the weight in liquid equals the weight of a volume of the liquid, which equals the volume of peas. Industry grade standards may be based on specific gravity values (Scott Rambo, personal communication, Dean Foods, Rockford, IL).

Suggested standards for frozen peas:

Fancy, 1.072 and lower
Standard, 1.073–1.084
Substandard, 1.085 and higher

Whole kernel corn can be assayed similarly with the following specific gravity standards:

Fancy, 1.080–1.118
Reject immature, 1.079 and lower
Reject overmature, 1.119 and higher

8.5.3 Refractometry

Moisture in liquid sugar products and condensed milks can be determined using a Baumé hydrometer (solids), a Brix hydrometer (sugar content), gravimetric means, or a refractometer. If it is performed correctly and no crystalline solids are evident, the refractometer procedure is rapid and surprisingly accurate (AOAC Method 9.32.14C, for solids in syrups). The refractome-

ter has been valuable in determining the soluble solids in fruits and fruit products (AOAC Method 932.12; 976.20; 983.17).

The refractive index of an oil, syrup, or other liquid is a dimensionless constant that can be used to describe the nature of the food. While some refractometers are designed only to provide results as refractive indices, others, particularly hand-held, quick-to-use units, are equipped with scales calibrated to read percent solids, percent sugars, and the like, depending on the products for which they are intended. Tables are provided with the instruments to convert values and adjust for temperature differences. Refractometers are used not just on the laboratory bench or as hand-held units. Refractometers can be installed in a liquid processing line to monitor the °Brix of products such as carbonated soft drinks, dissolved solids in orange juice, and percent solids in milk (12).

When a beam of light is passed from one medium to another and the density of the two differs, then the beam of light is bent or refracted. Bending of the light beam is a function of the media and the sines of the angles of incidence and refraction at any given temperature and pressure, and is thus a constant (Fig. 8-9). The refractive index (η) (RI) is a ratio of the sines of the angles:

$$\eta = \frac{\text{sine incident ray angle}}{\text{sine refracted ray angle}} \quad [12]$$

All chemical compounds have an index of refraction. Therefore, this measurement can be used for the qualitative identification of an unknown compound by comparing its RI with literature values. RI varies with concentration of the compound, temperature, and wavelength of light. Instruments are designed to give a reading by passing a light beam of a specific wavelength through a glass prism into a liquid, the sample. Bench-top or hand-held units use Amici prisms to obtain the D line of the sodium spectrum or 589 nm from white light. Whenever refractive indices of standard fluids are given, these are prefaced with $\eta^{20}_D = a$ value from 1.3000 to 1.7000. The Greek letter η is the symbol for refractive index; the 20 refers to temperature in °C; and D is the wavelength of the light beam—the D line of the sodium spectrum.

Bench-top instruments are more accurate compared to hand-held units mainly because of temperature control (Fig. 8-10). These former units have provisions for water circulation through the head where the prism and sample meet. Abbe refractometers are the most popular for laboratory use. Care must be taken when cleaning the prism surface following use. Wipe the contact surface clean with lens paper and rinse with distilled water and then ethanol. Close the prism chamber and cover the instrument with a bag when not in use to protect the delicate prism surface from dust or other debris that might lead to scratches and inaccuracy.
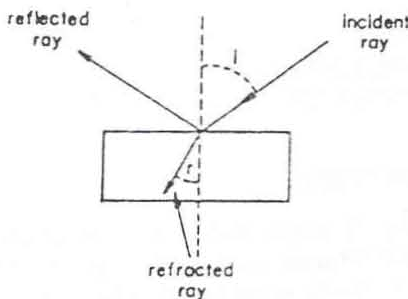
8-9 figure　Reflection and refraction concepts of refractometry.
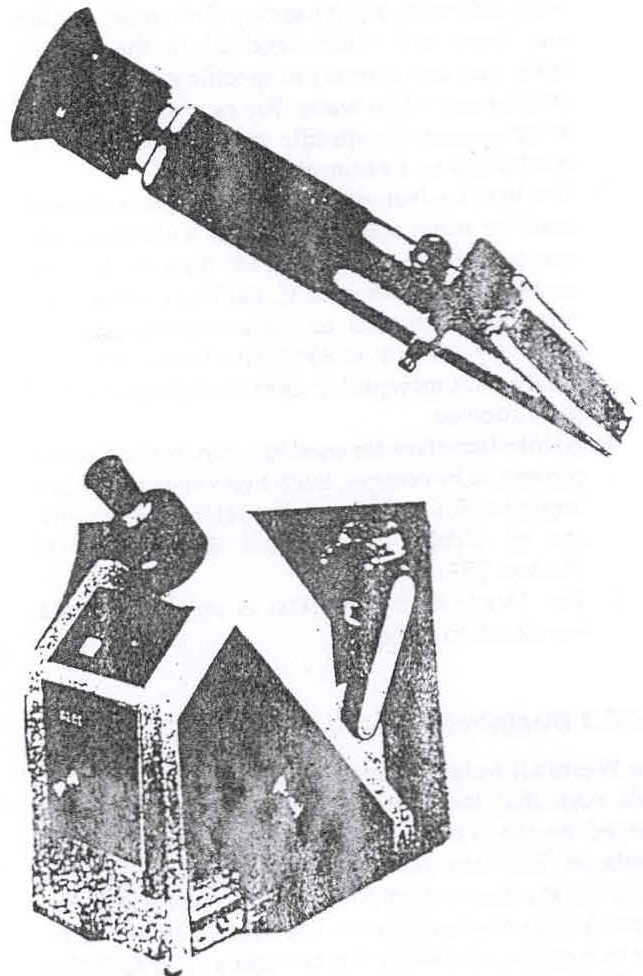
8-10 figure　Hand-held refractometer and Abbe refractometer. (Courtesy of Cole–Parmer Instrument Company, Vernon Hills, IL.)

The fact that the refractive index of a solution increases with concentration has been exploited in the analysis of total soluble solids of carbohydrate-based foods such as sugar syrups, fruit products, and tomato products. Because of this use, refractometers are calibrated in °Brix (g of sucrose/100 g of sample), which is equivalent to percentage sucrose or a wt/wt basis. Refractive index measurements are used widely to approximate sugar concentration in foods, even though values are accurate only for pure sucrose solutions.

8.5.4 Infrared Analysis

Infrared spectroscopy (see Chapter 27) has attained a primary position in monitoring the composition of food products during and following processing (13). It has a wide range of food applications and has proven successful in the laboratory, at-line, and on-line. Infrared spectroscopy measures the absorption of radiation (near- or mid-infrared) by molecules in foods. Different frequencies of infrared radiation are absorbed by different functional groups characteristic of the molecules in food. Similar to the use of ultraviolet (UV) or visible (Vis) light in UV-Vis spectroscopy, a sample is irradiated with a wavelength of infrared light specific for the constituent to be measured. The concentration of that constituent is determined by measuring the energy that is reflected or transmitted by the sample, which is inversely proportional to the energy absorbed. Infrared spectrometers must be calibrated for each analyte to be measured and the analyte must be informly distributed in the sample.

For water, near-infrared (NIR) bands (1400–1450, 1920–1950 nm) are characteristic of the –OH stretch of the water molecule and can be used to determine the moisture content of a food. NIR has been applied to moisture analysis of a wide variety of food commodities.

The use of mid-infrared milk analyzers to determine total solids in milk (AOAC Method 972.16) is covered in Chapter 27 of this text. The midrange spectroscopic method does not yield moisture or solids results except by computer calculation because these instruments do not monitor at wavelengths where water absorbs. The instrument must be calibrated using a minimum of eight milk samples that were previously analyzed for fat (F), protein (P), lactose (L), and total solids (TS) by standard methods. Then, a mean difference value, a, is calculated for all samples used in calibration:

$$a = \Sigma(TS - F - P - L)/n \qquad [13]$$

where:

a = solids not measurable by the F, P, and L methods

n = number of samples
F = fat percentage
P = protein percentage
L = lactose percentage
TS = total solids percentage

Total solids then can be determined from any infrared milk analyzer results by using the formula

$$TS = a + F + P + L \qquad [14]$$

The a value is thus a standard value mathematically derived. Newer instruments have the algorithm in their computer software to ascertain this value automatically.

8.5.5 Freezing Point

When water is added to a food product, many of the physical constants are altered. Some properties of solutions depend on the number of solute particles as ions or molecules present. These properties are vapor pressure, freezing point, boiling point, and osmotic pressure. Measurement of any of these properties can be used to determine the concentration of solutes in a solution. However, the most commonly practiced assay of this variety for milk is the change of the freezing point value. It has economic importance with regard to both raw and pasteurized milk. The freezing point of milk is its most constant physical property. The secretory process of the mammary gland is such that the osmotic pressure is kept in equilibrium with blood and milk. Thus, with any decrease in the synthesis of lactose, there is a compensating increase in the concentrations of Na^+ and Cl^-. While termed a physical constant, the freezing point varies within narrow limits, and the vast majority of samples from individual cows fall between –0.525 and –0.565°H (temperature in °H or Hortvet) (–0.503°C and –0.541°C). The average is very close to –0.540°H (–0.517°C). Herd or bulk milk will exhibit a narrower range unless the supply was watered intentionally or accidentally. Hortvet is the surname of the inventor of the first freezing point apparatus used for many years before automated equipment forced its obsolescence. All values today are given in °C by agreement. The following is used to convert °H to °C, or °C to °H (5, 6):

$$°C = 0.9623°H - 0.0024 \qquad [15]$$

$$°H = 1.03916°C + 0.0025 \qquad [16]$$

The principal utility of freezing point is to measure for added water. However, the freezing point of milk can be altered by mastitis infection in cows and souring of milk. In special cases, nutrition and environment of the cow, stage of lactation, and processing operations for the milk can affect the freezing point. If the solute

remains constant in weight and composition, the change of the freezing point varies inversely with the amount of solvent present. Therefore, we can calculate the percent H_2O added:

$$\% \; H_2O \; added = \frac{0.540 - T}{0.540} \times 100 \qquad [17]$$

where:

0.540 = freezing point in °H of all milk entering a plant

T = freezing point in °H of a sample

The AOAC cryoscopic method for water added to milk (AOAC Method of 961.07) assumes a freezing point for normal milk of −0.550°H (−0.527°C). The Food and Drug Administration will reject all milk with freezing points above −0.525°H (−0.503°C). Since the difference between the freezing points of milk and water is slight and since the freezing point is used to calculate the amount of water added, it is essential that the method be as precise as possible. The thermister used can sense temperature change to 0.001°H (0.001°C). The general technique is to supercool the solution and then induce crystallization by a vibrating reed. The temperature will rise rapidly to the freezing point or eutectic temperature as the water freezes. In the case of pure water, the temperature remains constant until all the water is frozen. In the case of milk, the temperature is read when there is no further temperature rise.

Instrumentation available is the old Hortvet cryoscope and the new Advanced Instruments (Fig. 8-11) and Fiske cryoscopes. Time required for the automated instruments is 1–2 min per sample using a prechilled sample.

8.6 WATER ACTIVITY

Water content alone is not a reliable indicator of food stability, since it has been observed that foods with the same water content differ in their perishability (14). This is at least partly due to differences in the way that water associates with other constituents in a food. Water tightly associated with other food constituents is less available for microbial growth and chemical reactions to cause decomposition. Water activity (a_w) is a better indication of food perishability than is water content. However, it is also an important quality factor for organoleptic properties such as hard/soft, crunchy/chewy, and the like. Water activity is defined as follows:

$$a_w = \frac{P}{P_0} \qquad [18]$$

$$a_w = \frac{ERH}{100} \qquad [19]$$
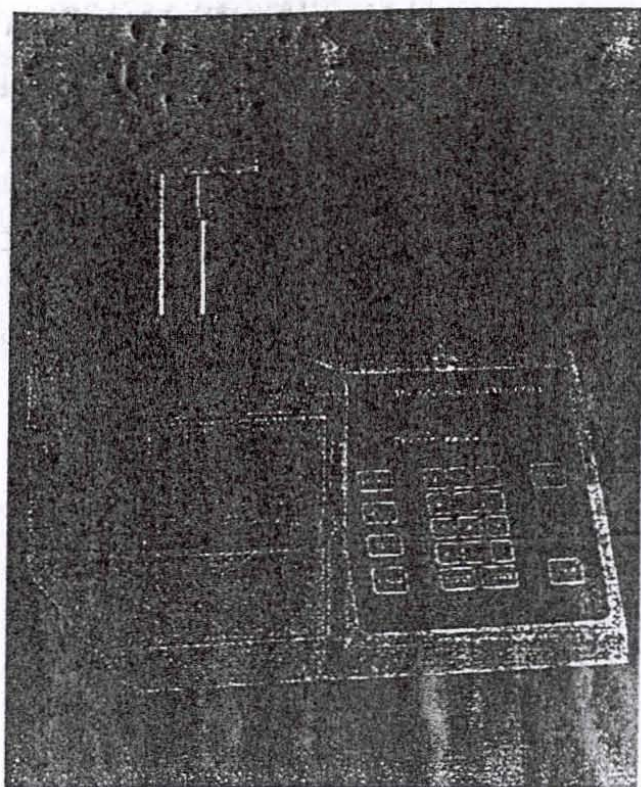
8-11 figure A model 4D3 Advanced Instruments cryoscope for freezing point determination in milk. (Courtesy of Advanced Instruments, Inc., Norwood, MA.)

where:

a_w = water activity
P = partial pressure of water above the sample
P_0 = vapor pressure of pure water at the same temperature (specified)
ERH = equilibrium relative humidity surrounding the product

There are various techniques to measure a_w. A commonly used approach relies on measuring the amount of moisture in the equilibrated headspace above a sample of the food product, which correlates directly with sample a_w. A sample for such analysis is placed in a small closed chamber at constant temperature, and a relative humidity sensor is used to measure the ERH of the sample atmosphere after equilibration. A simple and accurate variation of this approach is the chilled mirror technique, in which the water vapor in the headspace condenses on the surface of a mirror that is cooled in a controlled manner. The dew point is determined by the temperature at which condensation takes place, and this determines the relative humidity in the headspace. Two other general approaches to measuring a_w are (1) using the sample freezing point depression and moisture content to calculate a_w and (2)

equilibrating a sample in a chamber held at constant relative humidity (by means of a saturated salt solution) and then using the water content of the sample to calculate a_w (14).

8.7 COMPARISON OF METHODS

8.7.1 Principles

Oven drying methods involve the removal of moisture from the sample and then a weight determination of the solids remaining to calculate the moisture content. Nonwater volatiles can be lost during drying, but their loss is generally a negligible percentage of the amount of water lost. Distillation procedures also involve a separation of the moisture from the solids, but the moisture is quantitated directly by volume. Karl Fischer titration is based on chemical reactions of the moisture present, reflected as the amount of titrant used.

Dielectric and conductivity methods are based on electrical properties of water. Hydrometric methods are based on the relationship between specific gravity and moisture content. The refractive index method is based on how water in a sample affects the refraction of light. Near-infrared analysis of water in foods is based on measuring the absorption at wavelengths characteristic of the molecular vibration in water. Freezing point is a physical property of milk that is changed by a change in solute concentration.

8.7.2 Nature of Sample

While most foods will tolerate oven drying at high temperatures, some foods contain volatiles that are lost at such temperatures. Some foods have constituents that undergo chemical reactions at high temperatures to generate or utilize water or other compounds, to affect the calculated moisture content. Vacuum oven drying at reduced temperatures may overcome such problems for some foods. However, a distillation technique is necessary for some food to minimize volatilization and decomposition. For foods very low in moisture or high in fats and sugars, Karl Fischer titration is often the method of choice. The use of a pycnometer, hydrometer, and refractometer requires liquid samples, ideally with limited constituents.

8.7.3 Intended Purposes

Moisture analysis data may be needed quickly for quality control purposes, and high accuracy may not be necessary. Of the oven drying methods, microwave drying, infrared drying, and the moisture analyzer technique are fastest. Some forced draft oven procedures require less than 1 hr drying, but most forced

draft oven and vacuum oven procedures require much longer. The electrical, hydrometric, refractive index, and infrared analysis methods are very rapid but often require correlation to less empirical methods. Oven drying procedures are official methods for a variety of food products. Reflux distillation is an AOAC method for chocolate, dried vegetables, dried milk, and oils and fats. Such official methods are used for regulatory and nutrition labeling purposes.

8.8 SUMMARY

The moisture content of foods is important to food processors and consumers for a variety of reasons. While moisture determination may seem simplistic, it is often one of the most difficult assays in obtaining accurate and precise results. The free water present in food is generally more easily quantitated as compared to the adsorbed moisture and the water of hydration. Some moisture analysis methods involve a separation of moisture in the sample from the solids and then quantitation by weight or volume. Other methods do not involve such a separation but instead are based on some physical or chemical property of the water in the sample. A major difficulty with many methods is attempting to remove or otherwise quantitate all water present. This often is complicated by decomposition or interference by other food constituents. For each moisture analysis method, there are factors that must be controlled or precautions that must be taken to ensure accurate and precise results. Careful sample collection and handling procedures are extremely important and cannot be overemphasized. The choice of moisture analysis method is often determined by the expected moisture content, nature of other food constituents (e.g., highly volatile, heat sensitive), equipment available, speed necessary, accuracy and precision required, and intended purpose (e.g., regulatory or in-plant quality control).

8.9 STUDY QUESTIONS

1. Identify five factors that one would need to consider when choosing a moisture analysis method for a specific food product.
2. Why is standardized methodology needed for moisture determinations?
3. What are the potential advantages of using a vacuum oven rather than a forced draft oven for moisture content determination?
4. In each case specified below, would you likely overestimate or underestimate the moisture content of a food product being tested? Explain your answer.
   a. hot air oven
      • particle size too large

- high concentration of volatile flavor compounds present
- lipid oxidation
- sample very hygroscopic
- alteration of carbohydrates (e.g., Maillard browning)
- sucrose hydrolysis
- surface crust formation
- splattering
- desiccator with dried sample not sealed properly

b. toluene distillation
- emulsion between water in sample and solvent not broken
- water clinging to condenser

c. Karl Fischer
- very humid day when weighing original samples
- glassware not dry
- sample ground coarsely
- food high in Vitamin C
- food high in unsaturated fatty acids

5. The procedure for an analysis for moisture in a liquid food product requires the addition of 1–2 ml of deionized water to the weighed sample in the moisture pan. Why should you add moisture to an analysis in which moisture is being determined?

6. A new instrument based on infrared principles has been received in your laboratory to be used in moisture analysis. Briefly describe the way you would ascertain if the new instrument would meet your satisfaction and company standards.

7. A technician you supervise is to determine the moisture content of a food product by the Karl Fischer method. Your technician wants to know what is this "Karl Fischer Reagent Water Equivalence" that is used in the equation to calculate percentage of water in the sample, why is it necessary, and how is it determined. Give the technician your answer.

8. You are fortunate to have available in your laboratory the equipment for doing moisture analysis by essentially all methods—both official and rapid quality control methods. For each of the food products listed below (with the purpose specified as rapid quality control or official), indicate (a) the name of the method you would use, (b) the principle (not procedure) for the method, (c) a justification for use of that method (as compared to using a hot air drying oven), and (d) two cautions in use of the method to ensure accurate results.

a. ice cream mix (liquid)—quality control
b. milk chocolate—official
c. spices—official
d. syrup for canned peaches—quality control
e. oat flour—quality control

8.10 PRACTICE PROBLEMS

1. As an analyst, you are given a sample of condensed soup to analyze to determine if it is reduced to the correct concentration. By gravimetric means, you find that the concentration is 26.54% solids. The company standard reads 28.63%. If the starting volume were 1000 gallons at 8.67

solids, and the weight is 8.5 pounds per gallon, how much more water must be removed?

2. Your laboratory just received several sample containers of peas to analyze for moisture content. There is a visible condensate on the inside of the container. What is your procedure to obtain a result?

3. You have the following gravimetric results: weight of dried pan and glass disc = 1.0376 g, weight of pan and liquid sample 4.6274 g, and weight of the pan and dried sample 1.7321 g. What was the moisture content of the sample and what is the percent solids?

Answers

1. The weight of the soup initially is superfluous information. By condensing the soup to 26.54% solids from 8.67% solids, the volume is reduced to 326.7 gallons [(8.67/26.54) × 1000]. You need to reduce the volume further to obtain 28.63% solids [(8.67/28.63) × 1000, or 302.8 gallons. The difference in the gallons obtained is 23.9, or the volume of water that must be removed from the partially condensed soup to comply with company standards (326.7–302.8).

2. This problem focuses on a real issue in the food processing industry—when do you analyze a sample and when don't you? It would appear that the peas have lost water that should be within the vegetable for correct results. You will need to grind the peas in a food mill or blender. If the peas are in a Mason jar or one that fits an Oster blender head, no transfer is needed. Blend the peas to a creamy texture. If a container transfer was made, then put the blended peas back into original container. Mix with the residual moisture to a uniform blend. Collect a sample for moisture analysis. You should note on the report form containing the results of the analysis that the pea samples had free moisture on container walls when they arrived.

3. Note Equations [2]–[4] in section 8.2.1.7. To use any of the equations, you must subtract the weight of the dried pan and glass disc. Then you obtain 3.5898 g of original sample and 0.6945 g when dried. By subtracting these results, you have water removed or 2.8953 g. Then (0.6945/3.5898) × 100 = 19.35% solids and (2.8953/3.5898) × 100 = 80.65% water.

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9 chapter

Ash Analysis

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9.1 INTRODUCTION

Ash refers to the inorganic residue remaining after either ignition or complete oxidation of organic matter in a foodstuff. A basic knowledge of the characteristics of various ashing procedures and types of equipment is essential to ensure reliable results. Three major types of ashing are available: dry ashing for the majority of samples, wet ashing (oxidation) for samples with high fat content (meats and meat products) as a preparation for elemental analysis, and low-temperature plasma dry ashing (also called simply plasma ashing or low-temperature ashing) for preparation of samples when volatile elemental analyses are conducted. A microwave system now is available for both dry and wet ashing. Most dry samples (i.e., whole grain, cereals, dried vegetables) need no preparation, while fresh vegetables need to be dried prior to ashing. High-fat products such as meats may need to be dried and fat extracted before ashing. Fruits and vegetables may be subjected to additional ashing procedures such as soluble ash in water and alkalinity of ash. The ash content of foods can be expressed on either a wet weight (as is) or on a dry weight basis. Alkalinity of ash is a useful measurement to determine the acid-base balance of foods and to detect adulteration of foods with minerals.

9.1.1 Definitions

Dry ashing refers to the use of a muffle furnace capable of maintaining temperatures of 500–600°C. Water and volatiles are vaporized and organic substances are burned in the presence of oxygen in air to CO_2, and oxides of N_2. Most minerals are converted to oxides, sulfates, phosphates, chlorides, and silicates. Elements such as Fe, Se, Pb, and Hg may partially volatilize with this procedure, so other methods must be used if ashing is a preliminary step for specific elemental analysis.

Wet ashing is a procedure for oxidizing organic substances by using acids and oxidizing agents or their combinations. Minerals are solubilized without volatilization. Wet ashing is often preferable to dry ashing as a preparation for specific elemental analysis. Nitric and perchloric acids are preferable, but a special perchloric acid hood is essential. This procedure *must be* conducted in a perchloric acid hood and caution must be taken when fatty foods are used.

Low-temperature plasma ashing refers to a specific type of dry ashing method whereby foods are oxidized in a partial vacuum by nascent oxygen formed by an electromagnetic field. Ashing occurs at a much lower temperature than with a muffle furnace, preventing volatilization of most elements. The crystalline structures usually remain intact.

Acid insoluble ash generally refers to insoluble mineral contaminants in foods. Soil minerals (largely silicates and opaline silica) soluble only in HBr or HF comprise the major portion of this ash.

Alkalinity of ash is a useful measurement to determine the acid-base balance of foods and to detect adulteration of foods with minerals.

9.1.2 Importance of Ash in Food Analysis

Ash content represents the total mineral content in foods. Determining the ash content may be important for several reasons. It is a part of the proximate analysis for nutritional evaluation. Ashing is the first step in the preparation of a food sample for specific elemental analysis. Because certain foods are high in particular minerals, ash content becomes important. We can usually expect a constant elemental content from the ash of animal products, but that from plant sources is variable.

9.1.3 Ash Contents in Foods

The average ash content of the various food groups is given in Table 9-1. The ash content of most fresh foods rarely is greater than 5%. Pure oils and fats generally contain little or no ash, products such as cured bacon may contain 6% ash, and dried beef may be as high as 11.6% (wet weight basis).

Fats, oils, and shortenings vary from 0.0 to 4.09% ash, while dairy products vary from 0.5 to 5.1%. Fruits, fruit juice, and melons contain 0.2–0.6% ash, while dried fruits are higher (2.4–3.5%). Flours and meals vary from 0.3 to 1.4% ash. Pure starch contains 0.3% and wheat germ 4.3% ash. It would be expected that grain and grain products with bran would tend to be higher in ash content than such products without bran. Nuts and nut products contain 0.8–3.4% ash, while meat, poultry, and seafoods contain 0.7–1.3% ash.

9.2 METHODS

Principles, materials, instrumentation, general procedures, and applications are described below for various ash determination methods. Refer to methods cited for detailed instructions of the procedures.

9.2.1 Sample Preparation

It cannot be overemphasized that the small sample used for ash, or other determinations, needs to be very carefully chosen so that it represents the original materials. A 2–10 g sample generally is used for ash determination. For that purpose, milling, grinding, and the

9-1 table	Ash Content of Selected Foods

Food Item	Percent Ash (wet weight basis)
Cereals, bread, and pasta	
Rice, brown, long-grain, raw	1.5
Corn meal, whole-grain, yellow	1.1
Hominy, canned, white	0.9
White rice, long-grain, regular, raw, enriched	0.6
Wheat flour, whole-grain	1.6
Macaroni, dry, enriched	0.7
Rye bread	2.5
Dairy products	
Milk, whole, fluid	0.7
Evaporated milk, whole	1.6
Butter, with salt	2.1
Cream, fluid, half and half	0.7
Margarine, hard, regular, soybean	2.0
Yogurt, plain, low fat	0.7
Fruits and vegetables	
Apples, raw, with skin	0.3
Bananas, raw	0.8
Cherries, sweet, raw	0.5
Raisins	1.8
Potatoes, raw, skin	1.6
Tomatoes, red, ripe, raw	0.4
Meat, poultry, and fish	
Eggs, whole, raw, fresh	0.9
Fish fillet, battered or breaded, and fried	2.5
Pork, fresh, leg (ham), whole, raw	0.9
Hamburger, regular, single patty, plain	1.7
Chicken, broilers or fryers, breast meat only, raw	1.0
Beef, chuck, arm pot roast, raw	0.9

From USDA Nutrient Database for Standard Reference. Release 11-1 (August 1997)
http://www.nal.usda.gov/fnic/cgi-bin/nut_search.pl

like probably will not alter the ash content much; however, if this ash is a preparatory step for specific mineral analyses, contamination by microelements is of potential concern. Remember, most grinders and mincers are of steel construction. Repeated use of glassware can be a source of contaminants as well. The water source used in dilutions also may contain contaminants of some microelements. Distilled–deionized water should be used.

9.2.1.1 Plant Materials

Plant materials are generally dried by routine methods prior to grinding. The temperature of drying is of little consequence for ashing. However, the sample may be used for multiple determinations—protein, fiber, and so on—which require consideration of temperature for drying. Fresh stem and leaf tissue probably should be dried in two stages (i.e., first at a lower temperature of 55°C, then a higher temperature) especially to prevent artifact lignin. Plant material with 15% or less moisture may be ashed without prior drying.

9.2.1.2 Fat and Sugar Products

Animal products, syrups, and spices require treatments prior to ashing because of high fat and moisture (spattering, swelling) or high sugar content (foaming) that may result in loss of sample.

Meats, sugars, and syrups need to be evaporated to dryness on a steam bath or with an infrared (IR) lamp. One or two drops of olive oil (which contains no ash) are added to allow steam to escape as a crust is formed on the product.

Smoking and burning may occur upon ashing for some products (e.g., cheese, seafood, spices). Allow this smoking and burning to finish slowly by keeping the muffle door open prior to the normal procedure. A

sample may be ashed after drying and fat extraction. In most cases, mineral loss is minimal during drying and fat extraction. Under no circumstances should fat-extracted samples be heated until all the ether has been evaporated.

9.2.2 Dry Ashing

9.2.2.1 Principles and Instrumentation

Dry ashing is incineration at high temperature (525°C or higher). Incineration is accomplished with a muffle furnace. Several models of muffle furnaces are available, ranging from large-capacity units requiring either 208 or 240 voltage supplies to small bench-top units utilizing 110-volt outlets.

Crucible selection becomes critical in ashing because type depends upon the specific use. Quartz crucibles are resistant to acids and halogens, but not alkali, at high temperatures. Vycor® brand crucibles are stable to 900°C, but Pyrex® Gooch crucibles are limited to 500°C. Ashing at a lower temperature of 500-525°C may result in slightly higher ash values because of less decomposition of carbonates and loss of volatile salts. Porcelain crucibles resemble quartz crucibles in their properties but will crack with rapid temperatures changes. Porcelain crucibles are relatively inexpensive and usually the crucible of choice. Steel crucibles are resistant to both acids and alkalies and are inexpensive, but they are composed of chromium and nickel, which are possible sources of contamination. Platinum crucibles are very inert and are probably the best crucibles but they are currently far too expensive for routine use for large numbers of samples.

All crucibles should be marked for identification. Marks on crucibles with a felt-tip marking pen will disappear during ashing in a muffle furnace. Laboratory inks scribed with a steel pin are available commercially. Crucibles also may be etched with a diamond point and marked with a 0.5 M solution of FeCl, in 20% HCl. An iron nail dissolved in concentrated HCl forms a brown goo that is a satisfactory marker. The crucibles should be fired and cleaned prior to use.

The *advantages* of conventional dry ashing are that it is a safe method, it requires no added reagents or blank subtraction, and little attention is needed once ignition begins. Usually a large number of crucibles can be handled at once, and the resultant ash can be used for such other analyses as most individual elements, acid insoluble ash, and water-soluble and insoluble ash. The *disadvantages* are the length of time required (12-18 hr, or overnight) and expensive equipment. There will be a loss of the volatile elements and interactions between mineral components and crucibles. Volatile elements at risk of being lost include As, B, Cd, Cr, Cu, Fe, Pb, Hg, Ni, P, V, and Zn.

9.2.2.2 Procedures

AOAC International has several dry ashing procedures (e.g., AOAC Methods 900.02 A or B, 920.117, 923.03) for certain individual foodstuffs.

The general procedure includes the following steps:

1. Weigh a 5-10 g sample into a tared crucible. Predry if the sample is very moist.
2. Place crucibles in cool muffle furnace. Use tongs, gloves, and protective eyeware if the muffle furnace is warm.
3. Ignite 12-18 hr (or overnight) at about 550°C.
4. Turn off muffle furnace and wait to open it until the temperature has dropped to at least 250°C, preferably lower. Open door carefully to avoid losing ash that may be fluffy.
5. Using safety tongs, quickly transfer crucibles to a desiccator with a porcelain plate and desiccant. Cover crucibles, close desiccator, and allow crucibles to cool prior to weighing.

Note: Warm crucibles will heat air within the desiccator. With hot samples, a cover may bump to allow air to escape. A vacuum may form on cooling. At the end of the cooling period, the desiccator cover should be removed gradually by sliding to one side to prevent a sudden inrush of air. Covers with a ground glass sleeve or fitted for a rubber stopper allow for slow release of a vacuum.

The ash content is calculated as follows:

$$\% \text{ ash (dry basis)} = \frac{\text{wt after ashing} - \text{tare wt of crucible}}{\text{original sample wt} \times \text{dry matter coefficient}} \times 100 \quad [1]$$

where:

$$\text{dry matter coefficient} = \% \text{ solids}/100$$

For example, if corn meal is 87% dry matter, the dry matter coefficient would be 0.87. If ash is calculated on an as-received or wet-weight basis (includes moisture), delete the dry matter coefficient from the denominator. If moisture was determined in the same crucible prior to ashing, the denominator becomes (dry sample wt − tared crucible wt).

9.2.2.3 Special Applications

Some of the AOAC procedures recommend steps in addition to those listed previously. If carbon is still present following the initial incineration, several drops of H_2O or HNO_3 should be added; then the sample

should be re-ashed. If the carbon persists, such as with high-sugar samples, follow this procedure:

1. Suspend the ash in water.
2. Filter through ashless filter paper because this residue tends to form a glaze.
3. Dry the filtrate.
4. Place paper and dried filtrate in muffle furnace and re-ash.

Other suggestions that may be helpful and accelerate incineration:

1. High-fat samples should be extracted either by using the crude fat determination procedure or by burning off prior to closing the muffle furnace. Pork fat, for example, can form a combustible mixture inside the furnace and burn with the admission of oxygen if the door is opened.
2. Glycerin, alcohol, and hydrogen will accelerate ashing.
3. Samples such as jellies will spatter and can be mixed with cotton wool.
4. Salt-rich foods may require a separate ashing of water-insoluble components and salt-rich water extract. Use a crucible cover to prevent spattering.
5. An alcoholic solution of magnesium acetate can be added to accelerate ashing of cereals. An appropriate blank determination is necessary.

9.2.3 Wet Ashing

9.2.3.1 Principle, Materials, and Applications

Wet ashing is sometimes called wet oxidation or wet digestion. Its primary use is preparation for specific mineral analysis and metallic poisons.

There are several *advantages* to using the wet ashing procedure. Minerals will usually stay in solution, and there is little or no loss from volatilization because of the lower temperature. The oxidation time is short and requires a hood, hot plate, and long tongs, plus safety equipment.

The *disadvantages* of wet ashing are that it takes virtually constant operator attention, corrosive reagents are necessary, and only small numbers of samples can be handled at any one time. All work needs to be carried out in a special fume hood that may be washed. Those hoods are generally called perchloric acid hoods.

Unfortunately, a single acid used in wet ashing does not give complete and rapid oxidation of organic material. Nitric acid with either sulfuric or perchloric acids and potassium chlorate or sulfate are used in varying combinations. Different combinations are rec-

ommended for different samples. Sulfur and nitric oxides are expelled for complete oxidation. The nitric-perchloric combination is generally faster than the sulfuric-nitric procedure. Perchloric acid has a tendency to explode, so a special perchloric acid hood that has wash-down capabilities is recommended. The hood does not contain plastic or glycerol-base caulking compounds.

9.2.3.2 Procedures

The following wet ash procedure for nitric-perchloric oxidation is similar to that of the AOAC Method 975.03 for metals in plants:

1. A dried, ground 1-g sample is accurately weighed into a 150-ml Griffin beaker.
2. Add 10 ml HNO_3 and allow to soak. If the material has a high fat content, allow it to soak overnight.
3. Add 3 ml of 60% $HClO_4$. (*Precaution:* Place a beaker under pipette tip during transport) and slowly heat on a hot plate up to 350°C until frothing stops and HNO_3 is almost evaporated.
4. Continue boiling until perchloric reaction occurs (copious fumes), and then place watch glass on beaker. Sample should become colorless or light straw in color. *Do not let liquid in beaker reduce to dryness.*
5. Remove beaker from hot plate and let cool.
6. Wash watch glass with a minimum of distilled, deionized water and add 10 ml 50% HCl.
7. Transfer to appropriate volumetric flask (usually 50 ml) and dilute with distilled, deionized water.
8. Start wash-down procedure for hood after last sample.

An alternate procedure (i.e., as a preparation for iron analysis in meats) that could be used would be to use a 2-g sample boiled in 30 ml HNO_3 on a 350°C hotplate until 10 ml remain. Then add 10 ml of 60% perchloric acid and proceed as in step 4 above. Dilute to 100 ml in a volumetric flask following oxidation.

The wet ashing technique described above is very hazardous. Precautions for its use are found in the AOAC methods under "Safe Handling of Special Chemical Hazards." Perchloric acid interferes in the assay for iron by reacting with iron in the sample to form ferrous perchlorate, which forms an insoluble complex with the o-phenanthrolene in the procedure. It should not interfere with atomic absorption spectrophotometry.

The following procedure for a modified dry-wet ash oxidation may be used. It is listed under "Minerals

in Ready-to-Feed Milk-Based Infant Formula" (AOAC Method 985.35).

1. Evaporate moist samples (25–50 ml) at 100°C overnight or in a microwave drying oven.
2. Heat on a hot plate until smoking ceases.
3. Ash at 525°C for 3–5 hr.
4. Cool and wet with deionized distilled water plus 0.5–3.0 ml HNO_3.
5. Dry on a hot plate or steam bath and incinerate at 525°C for 1–2 hr.
6. Weigh sample after cooling in a desiccator.
7. Repeat steps 4 and 5 if carbon persists. (*Caution:* Some K may be lost with repeated ashing.)

9.2.4 Low-Temperature Plasma Ashing

9.2.4.1 Principles and Instrumentation

The equipment used for low-temperature plasma ashing consists of a glass system with a variable number of chambers for samples that may be evacuated by a vacuum pump. A small amount of oxygen is introduced that is broken into nascent oxygen by a radiofrequency electromagnetic field generator. A variable power frequency adjusts the rate of incineration. Air may be introduced as a gentler incineration procedure to preserve microscopic and structural components such as calcium oxalate crystals in various leaf tissues.

9.2.4.2 Procedures

The specific procedures for each type of low-temperature plasma ashing instrument may vary. The operator's manual should be consulted for proper operation.

The low-temperature plasma ashers usually contain two or more separate glass chambers with glass boats for holding samples. Intact or ground material is placed in individual boats, which are inserted into individual chambers. The chambers are sealed and a vacuum is applied. Once a vacuum is satisfactory (1 Torr or less), a small flow of oxygen or air is introduced into the system while maintaining a specific minimum vacuum. The frequency generator then is activated at a frequency slightly less than 14 MHz and adjusted by the amount of wattage applied (50–200 watts) to control the rate of incineration. Some models contain shaking devices to stir the sample. The progress of ashing may be viewed through the chambers.

The instrument is not without operational problems. These are usually due to leaks in the vacuum system. Either the seals around the chambers develop a leak and need to be replaced or breaks occur in the T-joints (usually plastic in the vacuum system). Those joints need to be replaced by glass material.

9.2.4.3 Applications

Low-temperature plasma ashing is a variation of dry ashing. The major *advantage* of this method is that there is less chance of losing trace elements by volatilization than with classical dry ashing techniques. The low temperature used with plasma ashers (150°C or less) generally allows the microscopic and crystalline structures to remain unaltered. The major *disadvantages* are small sample capacity and the expense of the equipment. However, it may be the equipment of choice under certain circumstances, especially for volatile salts.

9.2.5 Microwave Ashing

The CEM Corporation (Matthews, NC) has developed a series of instruments for dry and wet ashing as well as other microwave digestion series known as "microwave assisted chemistry." Programmed microwave wet digesters and muffle furnaces decrease time whether open or closed vessels are used for ashing a variety of samples. The systems allow for programming temperatures that can dehydrate, then ash and exhaust the system. For instance, dry ashing of flour takes 10–20 min. Wet ashing in a closed system is rapid and safe.

A comparative study (Zhang and Dotson) showed that dry ashing for 40 min using the microwave system was similar to the 4-hr time in a conventional muffle furnace. Twenty minutes were shown to be adequate for the plant material used except for Cu determinations, which needed 40 min to obtain similar results. The microwaving technique can speed up analysis significantly, but can be limited in the number of samples that can be processed at any one time.

9.2.6 Other Ash Measurements

9.2.6.1 Soluble and Insoluble Ash in Water

These measurements are an index of the fruit content of preserves and jellies. A lower ash in the water-soluble fraction is an indication that extra fruit is added to fruit and sugar products.

Use the following procedure to measure soluble and insoluble ash:

1. Weigh the total ash.
2. Add 10 ml distilled H_2O.
3. Cover the crucible and heat nearly to boiling.
4. Filter on ashless filter paper and rinse with hot distilled water several times.
5. Dry and re-ash filter paper at least 30 min.
6. Weigh and calculate as percent H_2O-insoluble ash.
7. Calculate soluble ash by subtracting insoluble

ash from total ash or dry the filtrate, re-ash, and weigh.

9.2.6.2 Ash Insoluble in Acid

This ash determination is a useful measure of the surface contamination of fruits and vegetables and wheat and rice coatings. Those contaminants are generally silicates and remain insoluble in acid, except HBr.

Use the following procedure:

1. Add 25 ml 10% HCl to total ash or H_2O-insoluble ash.
2. Cover and boil 5 min.
3. Filter on ashless filter paper and wash several times with hot distilled water.
4. Re-ash dried filter paper and residue at least 30 min.
5. Weigh and calculate as a percentage.

9.2.6.3 Alkalinity of Ash

The ash of fruits and vegetables is alkaline (Ca, Mg, K, Na), while that of meats and some cereals is acid (P, S, Cl). The alkalinity of ash has been used as a quality index of fruit and fruit juices. The salts of citric, malic, and tartaric acids yield carbonates upon combustion. Phosphates may interfere with this procedure. The procedure has been used for calculating acid–base balance, but its value in dietary calculations is questionable.

The following procedure is used to determine alkalinity of ash:

1. Place ash (total or water-insoluble ash) in platinum dish and accurately add 10 ml of 0.1 N HCl.
2. Add boiling H_2O if necessary and warm on a steam bath.
3. Cool and transfer to an Erlenmeyer flask.
4. Titrate the excess HCl with 0.1 N NaOH using methyl orange as an indicator.
5. Express in terms of ml of 1 N acid/100-g sample.

Alkalinity of insoluble ash can be determined by titrating directly with 0.1 N HCl using methyl orange. Express as described previously.

9.3 COMPARISON OF METHODS

Ashing by any one of three methodologies (dry ashing, wet ashing, low-temperature plasma ashing) requires expensive equipment, especially if a large number of

samples is analyzed. The muffle furnace may have to be placed in a heat room along with drying ovens and it requires a 220-volt outlet. It is important to make sure large furnaces of that type are equipped with a double-pole, single-throw switch. Heating coils are generally exposed, and care must be taken when taking samples in and out with metal tongs. Desk-top furnaces (110 volts) are available for fewer samples. Wet ashing by the nitric acid method or nitric–sulfuric acid combination requires a hood and corrosive reagents. It also requires constant operator attention. There are several digesters currently available for wet ashing, including microwave ovens and bomb colorimetry. Microwave technology currently is being assessed and compared to standard wet and dry ashing equipment and procedures. Those may be viable alternatives to a perchloric acid hood (which is expensive) even though the nitric-perchloric acid method is rapid. The low-temperature plasma asher requires a large vacuum pump in addition to the investment in the asher. Obviously, the type of further elemental analyses will dictate the equipment. Some micro- and most volatile elements will require special equipment and procedures. While wet oxidation and plasma ashing cause little volatilization, dry ashing will result in the loss of volatile elements. Refer to Chapter 10 for specific preparation procedures for elemental analyses.

9.4 SUMMARY

Three major types of ashing have been described: dry ashing, wet oxidation (ashing), and low-temperature plasma ashing. The procedure of choice depends upon the use of ash following its determination. Dry ashing, the method most commonly used, is based upon incineration at high temperatures in a muffle furnace. Except for certain elements, the residue may be used for further specific mineral analyses. Wet ashing (oxidation) is used in meat and meat products as a preparation for specific elemental analysis by simultaneously dissolving minerals and oxidizing all organic material. Low-temperature plasma ashers incinerate organic matter in a partial vacuum by forming nascent oxygen with a radiofrequency electromagnetic field generator. Highly volatile elements are preserved by this method. Wet ashing and low-temperature plasma ashing conserve volatile elements but are expensive, require operator time, and are limited to a small number of samples. Dry and wet ashing using microwave technology offers a new, rapid method that requires little additional equipment (special fume hood) or space (heat room). Three post-ashing procedures (soluble and insoluble ash in water, ash insoluble in acid, and alkalinity of ash) are special measurements for certain foods.

9.5 STUDY QUESTIONS

1. Identify four potential sources of error in the preparation of samples for ash analysis, and describe a way to overcome each.

2. You are determining the total ash content of a product using the dry ashing method. Your boss asks you to switch to a wet ashing method because he/she has heard it takes less time than dry ashing.
   a. Do you agree or disagree with your boss concerning the time issue, and why?
   b. Not considering the time issues, why might you want to continue using dry ashing, and why might you change to wet ashing?

3. Your lab technician was to determine the ash content of buttermilk by dry ashing. The technician weighed 5 g of buttermilk into one weighed platinum crucible, immediately put the crucible into the muffle furnace using a pair of all stainless steel tongs, and ashed the sample for 48 hr at 800°C. The crucible was removed from the muffle furnace and set on a rack in the open until it was cool enough to reweigh. Itemize the instructions you should have given your technician before beginning, so there would not have been the mistakes made as described above.

4. Differentiate low-temperature plasma ashing from conventional dry ashing with regard to principle and applications.

5. How would you recommend to your technician to overcome the following problems that could arise in dry ashing various foods?
   a. You seem to be getting volatilization of phosphorus, when you want to later determine the phosphorus content.
   b. You are getting incomplete combustion of a product high in sugar after a typical dry ashing procedure (i.e., the ash is dark colored, not white or pale gray).
   c. The typical procedure takes too long for your purpose. You need to speed up the procedure, but you do not want to use the standard wet ashing procedure.
   d. You have reason to believe the compound you want to measure after dry ashing may be reacting with the porcelain crucibles being used.
   e. You want to determine the iron content of some foods but cannot seem to get the iron solubilized after the dry ashing procedure.

6. Identify an advantage and disadvantage of using microwave wet digesters or microwave muffle furnaces compared to conventional units.

7. Explain two special ash measurements that can be useful for estimating the quality of fruits and fruit products.

9.6 PRACTICE PROBLEMS

1. A grain was found to contain 11.5% moisture. A 5.2146-g sample was placed into a crucible (28.5053 g tare). The ashed crucible weighed 28.5939 g. Calculate the percentage ash on (a) an as-received basis and (b) a dry-matter basis.

2. A vegetable (23.5000 g) was found to have 0.0940 g acid insoluble ash. What is the percentage acid insoluble ash?

3. You wish to have at least 100 mg ash from a cereal grain. Assuming 2.5% ash on average, how many grams of the grain should be weighed for ashing?

4. You wish to have a coefficient of variation (CV) below 5% with your ash analyses. The following ash data are obtained: 2.15%, 2.12%, 2.07%. Are these data acceptable, and what is the CV?

5. The following data were obtained on a sample of hamburger: sample wt, 2.034 g; wt after drying, 1.0781 g; wt after ether extraction, 0.4679 g; and wt of ash, 0.0233 g. What is the percentage ash on (a) a wet weight basis and (b) a fat-free basis?

Answers

1. (a) 1.70% (b) 1.92%. 2.04%. 3. 4 g. 4. Yes, 1.9%. 5. (a) 1.1%, (b) 1.57%.

9.7 RESOURCE MATERIALS

Analytical Methods Committee. 1960. Methods for the destruction of organic matter. *Analyst* 85:643–656. This report gives a number of methods for wet and dry combustion and their applications, advantages, disadvantages, and hazards.

AOAC International. 1995. *Official Methods of Analysis*, 16th ed. AOAC International, Gaithersburg, MD. This two-volume series contains the official methods for each specific food ingredient. It may be difficult for the beginning student to follow.

Aurand, L.W., Woods, A.E., and Wells, M.R. 1987. *Food Composition and Analysis*. Van Nostrand Reinhold, New York. The chapters that deal with ash are divided by foodstuffs. General dry procedures are discussed under each major heading.

Ockerman, H.W. 1991. *Food Science Source Book*, Part 2, 2nd ed. Van Nostrand Reinhold, New York.

Pomeranz, Y., and Meloan, C. 1994. *Food Analysis: Theory and Practice*, 3rd ed. Chapman & Hall, New York. Chapter 35 on ash and minerals gives an excellent narrative on ashing methods and is easy reading for a student in food chemistry. A good reference list of specific mineral losses is given at the end of the chapter. No stepwise procedures are given.

Marshall, R.T. (Ed.). 1992. *Standard Methods for the Examination of Dairy Products*, 16th ed. American Public Health Association, Washington, DC. This text gives detailed analytical procedures for ashing dairy products.

Smith, G.F. 1953. The wet ashing of organic matter employing hot concentrated perchloric acid. The liquid fire reaction. *Analytica Chimica Acta* 8:397–421. The treatise gives an in-depth review of wet ashing with perchloric acid. Tables on reaction times with foodstuffs and color reactions are informative. It is easy for the food scientist to understand.

Wooster, H.A. 1956. *Nutritional Data*, 3rd ed. H.J. Heinz Co., Pittsburgh, PA.

Zhang, H., and Dotson, P. Use of microwave muffle furnace for dry ashing plant tissue samples. Agricultural Testing and Research Laboratory, Navajo Agricultural Products Industry, Farmington, NM 87499.

10
chapter

Mineral Analysis

Deloy G. Hendricks

10.1 INTRODUCTION

Modern instrumentation has made it possible to quantitate an entire spectrum of minerals in one process. Some instruments are capable of detecting mineral concentrations in the parts per billion range. Instrumentation capable of such analysis is expensive and beyond the financial resources of many quality assurance laboratories. Large numbers of samples to be analyzed may justify the automation of some routine analyses and perhaps the expense of some of the modern pieces of equipment. The requirements for only occasional samples to be analyzed for a specific mineral, however, will not justify the initial costs of much instrumentation. This leaves the options of (1) sending samples out to certified laboratories for analysis or (2) utilizing one of the more traditional methods for analysis. Traditional methods generally require chemicals and equipment that are routinely available in an analytical laboratory.

In this chapter, the nutritional need for minerals, their roles in processed food, and methods for analysis of minerals involving gravimetric, titrimetric, and colorimetric procedures, and ion detective electrodes are described. Procedures for analysis of minerals of major nutritional or food processing concern are used for illustrative purposes. For additional examples of traditional methods currently in use, refer to references (1-3). Slight modifications of these methods are often needed for specific foodstuffs to minimize interferences or to be in the range of analytical accuracy. Methods for water, plant, or animal foodstuffs are reported here. For analytical requirements for specific foods see the *Official Methods of Analysis* of AOAC International, or other official methods.

10.1.1 Importance of Minerals in the Diet

Approximately 98% of the calcium and 80% of the phosphorus in the human body are found in the skeleton. Sodium, potassium, calcium, and magnesium are minerals involved in neural conduction and muscle contraction. Hydrochloric acid in the stomach greatly influences solubility and consequently absorbability of many minerals from foods in the diet. Calcium, phosphorus, sodium, potassium, magnesium, chlorine, and sulfur make up the dietary macro minerals, those minerals required at more than 100 mg per day by the adult (4). Each of these minerals has a specific function in the body. Physical malfunctions occur if these minerals are not provided in the diet on a regular basis.

An additional 10 minerals are required in milligram quantities per day and are referred to as trace minerals (4). These include iron, iodine, zinc, copper, chromium, manganese, molybdenum, fluoride, sele-

nium, and silica. Each of these minerals have specific biochemical roles in maintaining body functions. Iron, for example, is part of the hemoglobin and myoglobin molecules involved in oxygen transport to and within the cells.

There is also a group of minerals called ultra trace minerals that are being investigated for possible biological function, but that currently do not have clearly defined biochemical roles. These include vanadium, tin, nickel, arsenic, and boron.

Some mineral elements have been documented to be toxic to the body and should, therefore, be avoided in the diet. These include lead, mercury, cadmium, and aluminum. Essential minerals such as fluoride and selenium also are known to be harmful if consumed in excessive quantities, even though they do have beneficial biochemical functions at proper dietary levels.

Water, which is the nutrient required in the diet in the largest quantity (2-3 liters for an adult per day), may be obtained from drinking water, other beverages, foods, or as a by-product of metabolism of energy nutrients. Water as a beverage for drinking is seldom pure water but contains minerals, the composition of which depends on the water source. Thus drinking water may be a significant dietary source of some minerals. The introduction of fluoride in culinary water supplies, for example, has reduced the incidence of decayed, missing, and filled teeth in 10- to 12-year-old school children by about 70% in communities that have fluoridated their water supply at 0.7-1.0 ppm fluoride.

10.1.2 Minerals in Food Processing

Some minerals are inherent in natural foodstuffs. For example, milk is a good source of calcium, containing about 300 mg of calcium per 8-ounce cup. In some cases, salt is added in processing to decrease water activity and act as a preservative, thus increasing significantly the sodium content of products such as bacon, pickles, and Cheddar cheese. The enrichment law for flour requires that iron be replaced in white flour to the level at which it occurred naturally in the wheat kernel before removal of the bran. Fortification of foods has allowed addition of minerals into some foods above levels ever expected naturally. Prepared breakfast cereals often are fortified with minerals such as calcium, iron, and zinc, formerly thought to be limited in the diet. Fortification of salt with iodine has almost eliminated goiter in the United States.

Some processing of foods results in decreased mineral content. A large portion of the phosphorus, zinc, manganese, chromium, and copper found in a grain kernel is in the bran layer. When the bran layer is removed in processing, these minerals are removed. Direct acid cottage cheese is very low in calcium

because of the action of the acid causing the calcium bound to the casein to be freed and consequently lost in the whey fraction.

Water is an integral part of food processing. It is used for washing, rinsing, blanching, cooling, and as an ingredient in formulations. Microbiological safety of water used in food processing is very important. Also important, but generally not appreciated by the consuming public, is the mineral content of water used in food processing. Waters that contain excessive minerals can result in clouding of beverages. Textural properties of fruits and vegetables can be influenced by the "hardness" or "softness" of the water used in their processing. Thus, water quality is a major factor to be considered in the food processing industry.

The mineral content of foodstuffs is, therefore, important because of nutritional value, toxicological potential, and proper processing function and safety of some foods.

10.2 BASIC CONSIDERATIONS

Some sample preparation is required for traditional methods of mineral analysis. Methods used in sample preparation can remove interference for some analyses, add contaminants, or cause a loss of volatile elements. Proper handling of samples prior to the final analysis is very important for obtaining reliable analytical results for mineral content of foodstuffs.

10.2.1 Sample Preparation

Methods such as near infrared and neutron activation allow for mineral estimation without destruction of the carbon matrix of carbohydrates, fats, protein, and vitamins that make up foods. However, traditional methods generally require that the minerals be freed from this organic matrix in some manner. Chapter 9 describes the various methods used to ash foods in preparation for determination of specific mineral components of the food. Water samples are in a form such that minerals may be determined without further preparation.

A major concern in mineral analysis is contamination. Solvents such as water can contain significant quantities of minerals. Therefore, all procedures involving mineral analysis require the use of the purest reagents available. In some cases, the cost of ultrapure reagents may be prohibitive. When this is the case, the alternative is to always work with a reagent blank. A reagent blank is a sample of reagents used in the sample analysis, quantitatively the same as used in the sample but without any of the material being analyzed. This reagent blank, representing the sum of the mineral

contamination in the reagents, is then subtracted from the samples as they are quantitated.

10.2.2 Interferences

Factors such as pH, sample matrix, temperature, and other analytical conditions and reagents influence the ability of an analytical method to be used accurately to quantify a mineral. This is very clearly illustrated by the Parks, Hood, Hurwitz, and Ellis scheme of 12 inorganic elements as reviewed by Winton and Winton (5). In this scheme, molybdenum, manganese, iron, and phosphorus are determined on a dilute hydrochloride acid solution of a nitric acid–perchloric acid wet digest sample of food sample. An alkaline dithizone extraction then is used to separate sulfur, calcium, magnesium, potassium, and sodium for further individual analysis. An acid dithizone extraction then is used for separation of zinc, cobalt, and copper, which can be quantitated individually.

If interferences are suspected, it is a common practice to use a sample matrix for standard curve preparation. A sample matrix standard is made up of elements known to be in the sample at the same level at which they exist in the sample. For example, if a food sample was to be analyzed for calcium content, a solution of the known levels of sodium, potassium, magnesium, and phosphorus would be used to make up the calcium standards for developing the standard curve. If the major minerals known to exist are used to make up the background solution for the standards, then the standard solutions more closely resemble the samples in solution. If there are interferences among the major minerals, the impact in the standards and the samples should be similar if a sample matrix is used. For some minerals, there are specific interfering substances that must be suppressed for accurate analysis.

10.3 METHODS

10.3.1 Gravimetric Analysis

10.3.1.1 Principles

Insoluble forms of minerals are precipitated, rinsed, dried, and weighed to estimate mineral content using gravimetric procedures. Gravimetric analysis is based on the fact that the constituent elements in any pure compound are always in the same proportions by weight. For example, NaCl is always 39.3% sodium. In gravimetric analysis, the desired constituent is separated from contaminating substances by selective precipitation and then rinsing to minimize any adhering or trapped elements. The precipitated compound then is dried and weighed. The weight of the mineral ele-

ment is the same proportion of the weight of the compound as it is of the compound formed in the precipitated complex. Chloride, for example, is often precipitated as silver chloride. The silver chloride is rinsed, dried, and weighed. The weight of the chloride then can be calculated from the weight of the silver chloride because chloride is 24.74% of the molecular weight of silver chloride.

10.3.1.2 Procedure—Modified Gravimetric Determination of Calcium (AOAC Method 910.01)

Calcium can be determined by ashing a sample of known weight, solubilizing the ash in HCl, then adding ammonium oxalate to precipitate calcium as the oxalate. The precipitated calcium oxalate (CaC_2O_4) is washed repeatedly and then converted to calcium oxide (CaO) by a second ashing step. The weight of the CaO is used to calculate the calcium content of the sample (MW Ca/MW CaO = 0.7147).

10.3.1.3 Applications

Gravimetric procedures are best suited to large sample sizes and are limited generally to foods that contain large amounts of the element to be determined. Procedures using silver nitrate have been used to quantitate chloride. Most trace elements are in such low quantities in foods that gravimetric procedures are too insensitive to be of analytical value.

A disadvantage of the gravimetric procedure is the extra time involved in the second ignition where CaC_2O_4 is converted to CaO. Also, repeated washing of the CaC_2O_4 precipitate tends to cause some solubilization. However, coprecipitation of other minerals necessitates the rinsing steps.

10.3.2 EDTA Complexometric Titration

10.3.2.1 Principles

There are a number of carboxylic acids containing tertiary amines that form stable complexes with a variety of metal ions. Ethylenediaminetetraacetic acid (EDTA) is the most important of this class of reagents referred to as versenes. The disodium salt, usually written as Na_2H_2Y, is available in high purity as the dihydrate. Since EDTA has both donor nitrogen and donor oxygen atoms, it can form as many as six five-membered chelate rings and forms complexes with practically all metals except the alkali metals of group I.

In general, 1:1 complexes are formed between EDTA and metallic ions. Typical reactions could be summarized as:

$$m^{2+} + H_2Y^{2-} \rightarrow mY^{2-} + 2H^+ \qquad [1]$$

$$m^{3+} + H_2Y^{2-} \rightarrow mY^- + 2H^+ \qquad [2]$$

$$m^{4+} + H_2Y^2 \rightarrow mY + 2H^+ \qquad [3]$$
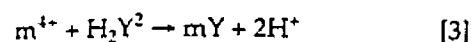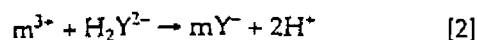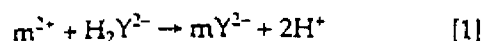
Obviously, pH will greatly influence the complex formation. The EDTA complexes are highly stable and therefore can be used for volumetric analysis.

10.3.2.2 Procedure—Calcium Determination Using EDTA Titration (AOAC Method 968.31)

Calcium content of foods can be determined by complexing calcium with EDTA in a titration procedure (Fig. 10-1). The standard curve should be established before running a sample to be certain of the endpoint color.

10.3.2.3 Applications

EDTA complexometric titration is suitable for fruits and vegetables and other foods that have calcium

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CALCIUM–EDTA TITRATION

Pipette an aliquot of dissolved ashed sample expected to contain 2–10 mg of calcium into a 250-ml beaker.

⇩

Dilute to 50 ml and adjust pH to 12.5–13.0 by dropwise addition of KOH–KCN solutions (dissolve 28 g KOH and 6.6 g KCN in 100 ml of d H_2O) while stirring with a magnetic stirrer.

⇩

Add 100 mg of ascorbic acid and about 250 mg of hydroxynaphthol blue indicator.

⇩

Titrate immediately with 0.01 M EDTA solution (dissolve 3.72 g of Na_2H_2 EDTA·2H_2O. 99+% purity, in d H_2O in a 1-liter vol. flask. Dilute to volume and mix). The endpoint in this titration is a deep blue end point.

Standard Curve

Prepare and titrate standard solutions containing 2.5, 5.0, 7.5, and 10.0 mg of calcium to develop a standard curve:

Dry primary standard grade calcium carbonate for 2 hr at 285°F.

⇩

Weigh out 2.5 g of the dried calcium carbonate and transfer quantitatively into a 1-liter vol. flask.

⇩

Dissolve the calcium carbonate in 50 ml of 3 N HCl.

⇩

Dilute to volume with d H_2O and thoroughly mix. Samples of 2.5, 5.0, 7.5 and 10 ml of this solution represent 2.5, 5.0, 7.5, and 10 mg of calcium, respectively.

10-1
figure

Procedure for calcium determination by EDTA titration. AOAC Method 968.31). [Adapted from (1).]

without appreciable magnesium or phosphorus. Phosphorus may be removed by passing the ashed material through an Omberlite IR-4B resin bed at pH 3.5 prior to adjusting the base for titration. Using calmagite as an indicator, magnesium content of the sample can be calculated by difference (AOAC Method 967.30).

10.3.3 Redox Reactions

10.3.3.1 Principles

The basis of many analytical methods is an oxidation–reduction reaction. The reaction of a substance with oxygen is defined as oxidation. Therefore, a reduction is the removal of oxygen. As we now know, oxidation is actually the removal of electrons from an atom, while reduction is the gain of electrons. Other reactions that do not involve oxygen involve the loss or gain of electrons. Any reaction that results in an increase in positive charge is termed oxidation, while any reaction that decreases positive charge is termed reduction whether or not oxygen is involved.

Since electrons cannot be created or destroyed in ordinary chemical reactions, any oxidation must be accompanied by a corresponding reduction. All oxidation–reduction reactions can be considered to be the reaction of an oxidizing agent with a reducing agent. Such reactions cause the oxidizing agent to be reduced and the reducing agent to be oxidized.

In some oxidation–reduction titrations, a colored reactant or product can act as the indicator. Permanganate ion is a deep purple, while manganous ion is a very pale pink. Thus, permanganate titrations have a built-in indicator.

10.3.3.2 Procedures

10.3.3.2.1 Calcium Determination Using Redox Titration (AOAC Method 921.01) Phosphates and magnesium tend to interfere with the analysis for calcium. Therefore, as described in the gravimetric procedure (section 10.3.1.2), calcium often is precipitated as an oxalate in the redox titration procedure to minimize the presence of interfering minerals in the final titration solution. As in the gravimetric method, the redox titration method has the disadvantage of requiring the precipitation and the washing of calcium oxalate. In the redox titration procedure, the precipitated and washed calcium oxalate then is solubilized in H_2SO_4, heated, and titrated with potassium permanganate to a slight pink endpoint. The volume and normality of the titrant are used to determine the calcium content of the sample.

10.3.3.2.2 Iron Determination Using Redox Reaction and Colorimetry (AOAC Method 944.02) There are a

number of organic compounds that effectively function as redox indicators. These compounds form stable colors that can be quantitated colorimetrically by measuring light absorbance at characteristic wavelengths.

Iron is quantitated by its ability to complex with organic compounds, resulting in formation of colored products proportional to the iron content (Fig. 10-2). All glassware must be acid washed and triple rinsed in distilled water to avoid iron contamination. Because many reagents contain small amounts of iron, it is important always to use a reagent blank for iron determinations.

10.3.3.3 Applications

Generally, redox reactions have been of limited use for quantitating metals in foods. Calcium, iron, copper, and iodine concentrations have been determined using this approach. Determination of iron in foods using this method appears to have some advantages over atomic absorption spectroscopy (see Chapter 28). Higher recovery from spiked samples and closer match to a wider variety of National Institute of Standards and Technology (formerly known as the National Bureau of Standards) samples have been observed for iron analyzed using this redox colorimetric method compared to atomic absorption spectroscopy.

Redox methodology is used widely for quantitation of elements and compounds in the food industry. For example, AOAC Method 990.28 uses a redox titration to determine sulfites in foods. With the current awareness of individual sensitivity to sulfites, the food service industry, in particular, is checking for sulfites on fresh produce. AOAC Method 967.21 uses the redox indicator 2, 6-dichloroindophenol to determine ascorbic acid by titration.

10.3.4 Precipitation Titration

10.3.4.1 Principles

When at least one product of a titration reaction is an insoluble precipitate, it is referred to as precipitation titrimetry. Few of the many gravimetric methods, however, can be adapted to yield accurate volumetric methods. Some of the major factors blocking the adaptation are (1) time to complete a reaction, resulting in a complete precipitation of the compound being formed; (2) failure of the reaction to yield a single product of definite composition; and (3) lack of an endpoint indicator for the reaction.

The potential of precipitation titration has resulted in at least two methods that are used widely in the food industry today. The Mohr method for chloride determination is based on the formation of an orange-col-

IRON—REDOX TITRATION

Weigh into a clean, dried crucible a food sample expected to contain 50–500 μg of iron.

↓

Add 10 ml of a glycerol–ethanol (1:1) mixture and dry over a low heat to avoid splattering.

↓

Ash for 24 hr at 600°C.

↓

After cooling add 1 ml conc. nitric acid and then evaporate to dryness.

↓

Return to the muffle furnace at 600°C for 1 hr to completely eliminate carbon particles.

⇓

Cool and add 5 ml of 6 N HCl to the ash.

⇓

Heat in a steam bath for 15 min.

↓

Filter through a hardened filter paper into a 100-ml vol. flask with at least three rinsings with hot d H_2O.

⇓

Dilute to volume after allowing to reach room temperature.

⇓

Pipette a 10-ml aliquot of the dissolved ash solution into a clean 25-ml vol. flask.

⇓

Add 1 ml of a 10% solution of hydroxylamine hydrochloride.

⇓

Allow to stand after mixing for a few minutes.

⇓

Add 5 ml of acetate buffer. (Made by dissolving 8.3 g of sodium acetate in 20 ml of water in a 100-ml vol. flask, adding 12 ml of acetic acid, and diluting to volume.)

⇓

Add as a color developing agent 1 ml of 0.1% orthophenanthroline or 2 ml of 0.1% α, α-dipyridyl solution.

⇓

Dilute to volume with mixing.

⇓

Allow to stand for 30 min.

⇓

Read absorbance at 510 nm.

Standard Curve

Make an iron standard stock solution of 100 ppm by dissolving 0.1 g of analytical grade iron wire in 20 ml of conc. HCl and diluting to 1 liter.

⇓

Prepare working standards by pipetting 0, 2.0, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 35.0, 40.0, and 45.0 ml of the standard stock solution plus 2 ml of conc. HCl into 100-ml vol. flasks and diluting to volume.

↓

10 ml of each of these working standards should be treated as the 10 ml of samples in the analytical procedure above.

↓

Plot the standard curve and use it to calculate iron concentration in your sample.

10-2 figure Procedure for iron determination using redox reaction and colorimetry. AOAC Method 944.02. [Adapted from (1).]

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ored solid, silver chromate after silver from silver nitrate has complexed with all the available chloride.

$$Ag^+ + Cl^- \rightarrow AgCl \text{ (until all } Cl^- \text{ is complexed)} \quad [4]$$

$$2Ag^+ + CrO_4^{2-} \rightarrow Ag_2CrO_4$$
(orange only after Cl^- is all complexed) [5]
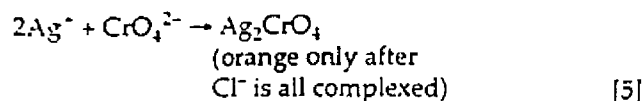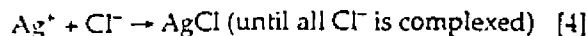
The Volhard Method is an indirect or back-titration method in which an excess of a standard solution of silver nitrate is added to a chloride-containing solution. The excess silver nitrate is then back-titrated using a standardized solution of potassium or ammonium thiocyinate with ferric ion as an indicator. In a back-titration such as the Volhard method, the excess silver is then back-titrated to calculate the amount of chloride that precipitated with the silver in the first steps of the reaction.

$$Ag^+ + Cl^- \rightarrow AgCl \text{ (until all } Cl^- \text{ is complexed)} \quad [6]$$

$$Ag^+ + SCN^- \rightarrow AgSCN \text{ (to quantitate silver}$$
not complexed with chloride) [7]

$$SCN^- + Fe^{+3} \rightarrow FeSCN \text{ (red when}$$
there is any SCN^- not complexed to Ag^+) [8]
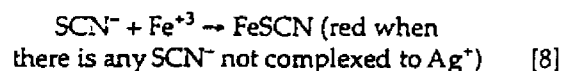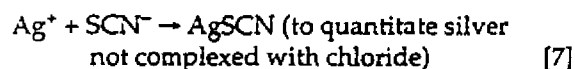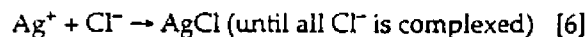
10.3.4.2 Procedures

10.3.4.2.1 Mohr Titration of Salt in Butter (AOAC Method 960.29) Salt in foods may be estimated by titrating the chloride ion with silver (Fig. 10-3). The orange endpoint in this reaction occurs only when all chloride ion is complexed, resulting in an excess of silver to form the colored silver chromate. The endpoint of this reaction is therefore at the first hint of an orange color. When preparing reagents for this assay, use boiled water to avoid interferences from carbonates in the water.

10.3.4.2.2 Volhard Titration of Chloride in Plant Material (AOAC Method 915.01) In the Volhard method (Fig. 10-4), water must be boiled to minimize errors due to interfering carbonates, since the solubility product of silver carbonate is greater than the solubility product of silver chloride. Once chloride is determined by titration, the chloride weight is multiplied by 1.648 to obtain salt weight, if salt content is desired.

10.3.4.3 Applications

Gravimetric titration methods are well suited for any foods that may be high in chlorides. Because of added salt in processed cheeses and meats, these products should certainly be considered for using this method to detect chloride; then salt content is estimated by calculation. The Quantab chloride titration used in AOAC Method 971.19 is an adaptation of the principles

SALT—MOHR TITRATION

Weigh about 5 g of butter into 250-ml Erlenmeyer flask and add 100 ml of boiling H_2O.

↓

Let stand 5–10 min with occasional swirling.

↓

Add 2 ml of a 5% solution of K_2CrO_4 in d H_2O.

↓

Titrate with 0.1 N $AgNO_3$ standardized as below until an orange-brown color persists for 30 sec.

Standardization of 0.1 N $AgNO_3$

Accurately weigh 300 mg of recrystallized dried KCl and transfer to a 250-ml Erlenmeyer flask with 40 ml of water.

↓

Add 1 ml of K_2CrO_4 solution and titrate with $AgNO_3$ solution until first perceptible pale red-brown appears.

↓

From the titration volume subtract the milliliters of the $AgNO_3$ solution required to produce the end point color in 75 ml of water containing 1 ml of K_2CaO_4.

↓

From the next volume of $AgNO_3$ calculate normality of the $AgNO_3$ as:

$$\text{Normality } AgNO_3 = \frac{mg\ KCl}{ml\ AgNO_3 \times 74.555\ g\ KCl/mole}$$

Calculating Salt in Butter

$$\text{Percent salt} = \frac{ml\ 0.1\ N\ AgNO_3 \times 0.585}{g\ of\ sample}$$

[0.585 = (58.5 g NaCl/mole)/100]

10-3 figure

Procedure of Mohr titration of salt in butter. AOAC Method 960.29. [Adapted from (1).]

involved in the Mohr titration methods. This adaptation allows for very rapid quantitation of salt in food products. The Quantab adaptation is accurate to ±10% over a range of 0.3–10% NaCl in food products.

10.3.5 Colorimetric Methods

10.3.5.1 Principles

In the visible region of the electromagnetic spectrum, certain wavelengths are absorbed and others are reflected from an object. The reflected wavelength range is the color we see. In the colorimetric methods, a chemical reaction must result in a stable color that develops rapidly and is the result of a single colored product. The color-forming reaction should be selective for the mineral being analyzed.

As a color intensity increases, less light is able to pass through a solution. As the light passes through a longer pathway of the solution, there is also less light transmitted. Beer's law, which defines these relationships, is explained in detail in Chapter 26.

With the ability to quantitate light transmitted through a solution, or conversely, light absorbed by a solution, it is possible to determine concentrations of reacting substances. This principle has been used to develop methods for determining concentration of many minerals.

10.3.5.2 Procedure—Determination of Phosphorus By Colorimetry (AOAC Method 986.24)

The color intensity of phosphomolybdovanadate can be quantitated spectrophotometrically as shown in Fig. 10-5. This is only one of many methods described using the phosphomolybdate reaction. This procedure has the advantage of producing a more stable color than most and therefore is preferred.

10.3.5.3 Applications

Colorimetry is used for a wide variety of minerals. The example of iron determination given as an example of oxidation–reduction reaction is quantitated using colorimetry. An oxidation–reduction reaction is, however, involved in the color development.

Some detergents contain phosphorus. It is necessary to thoroughly rinse all glassware carefully at least three times with distilled water to avoid contamination in determination of phosphorus by colorimetry.

10.3.6 Ion-Selective Electrodes

10.3.6.1 General Information

The concept of measuring [H^+] is considered in Chapter 7. One must question whether this application of potentiometry can be used in the measurement of other ions. It is only in recent years that much attention has been given to this question. Indeed, many electrodes have been developed for the direct measurement of various cations and anions, such as bromide, calcium, chloride, fluoride, potassium, sodium, and sulfide. There are even electrodes available for measuring dissolved gases, such as ammonia, carbon dioxide, and oxygen. While some of these methods are limited in their application due to interference from other ions, it often is possible to overcome this problem by pH adjustment, reduction of the interferent, or removal of the interferent by complexing or precipitation.

Varying the composition of the glass in a glass electrode is one means of changing the sensitivity of the glass membrane to other ions. An electrode membrane containing 71% SiO_2, 11% Na_2O, and 18% Al_2O_3 is sensitive to potassium.

A typical glass membrane sodium-indicating electrode operates in the range of 1–10^{-6} M or 23,000 to 0.023 ppm. Interferences from silver, lithium, potassium, and ammonium ions are a possibility. Response

SALT—VOLHARD TITRATION

Moisten 5-g sample in crucible with 20 ml of 5% Na_2CO_3 in water.

↓

Evaporate to dryness.

↓

Char on a hot plate under a hood until smoking stops.

↓

Combust at 500°C for 24 hr.

↓

Dissolve residue in 10 ml of 5 N HNO_3.

↓

Dilute to 25 ml with d H_2O.

↓

Titrate with standardized $AgNO_3$ solution (from the Mohr method) until white AgCl stops precipitating and then add a slight excess.

↓

Stir well, filter through a retentive filter paper, and wash AgCl thoroughly.

↓

Add 5 ml of a saturated solution of $FeNH_4(SO_4)_2 \cdot 12H_2O$ to the combined titrate and washings.

↓

Add 3 ml of 12 N HNO_3 and titrate excess silver with 0.1 N potassium thiocyanate.

Standardization of Potassium Thiocyanate Standard Solution

Determine working titer of the 0.1 N potassium thiocyanate standard solution by accurately measuring 40–50 ml of the standard $AgNO_3$ and adding it to 2 ml of $FeNH_4(SO_4)_2 \cdot 12H_2O$ indicator solution and 5 ml of 9 N HNO_3.

↓

Titrate with thiocyanate solution until solution appears pale rose after vigorous shaking.

Calculating Cl Concentration

Net volume of the $AgNO_3$ = Total volume $AgNO_3$ added − Volume titrated with thiocyanate
1 ml of 0.1 M $AgNO_3$ = 3.506 mg chloride

10-4 **figure**   Procedure for Volhard titration of chloride in plant material. AOAC Method 915.01. [Adapted from (1).]

time is less than 30 sec. Combination polymer-body sodium ion-selective electrodes also are available, a calomel reference half-cell being used in this system.

Solid-state ion-selective electrodes also are available. These electrodes do not use a glass-sensitive membrane. Instead, the active membrane consists of a single inorganic crystal treated with a rare earth. The fluoride electrode serves as a good example, consisting of a crystal of lanthanum chloride treated with europium, which permits ionic charge transport and lowered electrical resistance. Fluoride concentrations of 0.02 ppm may be detected with this electrode. Other commonly used solid-state ion-selective electrodes are available. For example, bromide can be detected at concentrations of 0.04 ppm and chloride at 0.178 ppm. Response time for all the solid-state electrodes is less than 30 sec. These electrodes also are subject to interferences from various anions.

In addition to the various glass membrane and solid-state electrodes, it should be noted that there are other types of these electrodes, such as precipitate-impregnated, liquid–liquid membrane, and even enzyme electrodes. The use of gas-sensing electrodes is also increasing. These electrodes possess a gas-permeable membrane and a combination pH electrode with

internal buffer solution. Upon passing through the membrane, the gas dissolves in a thin layer of buffer solution that surrounds the combination pH electrode. The dissolved gas causes the pH of the solution to change, and the combination electrode detects this change. Ammonia, carbon dioxide, sulfur dioxide, and oxygen can be measured by this type of electrode.

10.3.6.2 Activity Versus Concentration

In using ion-selective electrodes, the concept of activity versus concentration must be considered. Activity is a measure of chemical reactivity, while concentration is a measure of all forms (free and bound) of ions in solution. Due to interactions of ions with themselves and with the solvent, the effective concentration or activity is, in general, lower than the actual concentration. Activity and concentration are related by the following equation:

$$A = VC \qquad [9]$$

where:

A = activity
V = activity coefficient
C = concentration

PHOSPHORUS—COLORIMETRIC ASSAY

Ash a 2-g sample for 4 hr at 600°C.

⇓

Cool; add 5 ml of 6 N HCl and several drops of nitric acid.

⇓

Heat to dissolve the ash completely.

⇓

Cool and transfer to a 100-ml vol. flask and dilute to volume
with d H₂O.

⇓

Pipette an aliquot expected to contain 0.5–1.5 mg of phosphorus
into a 100-ml vol. flask.

⇓

Add 20 ml of molybdovanadate reagent. (This reagent is prepared
by dissolving 20 g of ammonium molybdate in 200 ml of hot H₂O,
then dissolving 1 g of ammonium meta vanadate in 125 ml of hot
H₂O to which when cooled is added 140 ml of conc. nitric acid.
The cooled molybdate and vanadate solutions are then combined
and diluted to 1 liter.)

⇓

Dilute the sample and the molybdovanadate reagent to 100 ml.

⇓

Allow the color to develop for 10 min.

⇓

Read the absorbance at 400 nm against a phosphorus
standard curve.

Preparation of Standard Curve

Make a stock standard solution of 2 mg P/ml by weighing 8.7674 g
of KH₂PO₄ that has been dried at 105°C for 2 hr.

⇓

Quantitatively transfer to a 1 liter vol. flask and add about 750 ml of
d H₂O to dissolve.

⇓

Dilute to volume with H₂O

⇓

Store refrigerated until use.

⇓

Make a working standard solution containing 0.1 mg
phosphorus/ml by diluting 50 ml of the stock solution to 1 liter
with d H₂O.

⇓

Transfer aliquots of the working standard solution of 0, 5, 8, 10,
and 15 ml to freshly rinsed 100-ml vol. flasks. (These represent 0,
0.5, 0.8, 1.0, and 1.5 mg of phosphorus, respectively.)

⇓

Add 20 ml of the molybdovanadate reagent to each flask
containing the standards.

⇓

Dilute to volume with H₂O and mix well.

⇓

Let flasks stand for 10 min to complete color development.

⇓

Read absorbance at 400 nm. Use the 0.0 standard (blank) to zero
the spectrophotometer.

10-5 figure   Procedure for determination of phosphorus by colorimetry. AOAC Method 986.24. [Adapted from (1).]

The activity coefficient is a function of ionic strength. Ionic strength is a function of the concentration of, and the charge on, all ions in solution.

By adjusting the ionic strength for all test samples and standards to a nearly constant level, the Nernst equation (see Chapter 7) can be used to relate electrode response to the concentration of the measured species. In practice, both samples and calibrating standards are adjusted to a high but constant ionic strength. An ionic-strength adjustment buffer is used for this purpose. It is a solution of neutral or noninterfering ions that raises the total ionic strength of the solution to the level at which the effects of other ions are canceled. These buffers also can be used to control pH, remove ionic interferences, and limit chemical interferences arising from association and complexation. Thus, to measure accurately the concentration of ionic species using an ion-selective electrode, the following requirements must be met:

1. Maintain a constant reference potential.
2. Operate a constant temperature.
3. Adjust ionic strength.
4. Adjust pH.
5. Remove electrode interferences.
6. Eliminate method interferences

10.3.6.3 Calibration Curves

In working with ion-selective electrodes it is common practice to develop a calibration curve. The two electrodes (indicator and reference) are immersed in a series of solutions of known concentration. The electrode potential (millivolts) developed in these standard solutions is recorded and plotted (on semilog paper) against the logarithms of the standard concentrations (Fig. 10-6). Upon analysis of a test sample, the observed millivolt reading for the electrode potential is used to determine the concentration by referring to the calibration curve.

The calibration curve has a linear region at which the electrode has a constant response to changes in concentration, fitting the Nernst equation ($E = E° - 0.059$ [ion]). Note also the nonlinear region of the curve at low concentrations. The total ionic strength and the concentrations of interfering ions are among the factors that determine the lowest level of activity that can be detected in practical applications. Examples of calibration curves for various ions are found in Fig. 10-7.

10.3.6.4 Other Ion-Selective Electrode Methodologies

Although a calibration curve is the most common means of using ion-selective electrodes, there are other applications. For example, in a titration the ISE may be employed to detect the equivalence point of the titration. The ISE may be responsive to either the sample species (S titration) or the titrant (T titration), the latter probably in more common use.

In the T titration, little change in electrode poten-

10-6 figure A typical calibration curve for ion-selective electrodes. [From (6), used with permission.]

10-8 figure A typical T-type titration. [From (6), used with permission.]

potential is determined. Then an aliquot containing a known concentration of the measured species is added (standard addition) to the sample, and a second measurement of electrode potential is determined. These measured values in electrode potential then may be used to determine the concentration of the active species in the original sample. This method may not require the use of an ionic strength adjustment buffer. It is of great value when only a few samples are to be measured and time does not permit the development of a calibration curve. It also eliminates complex unknown background effects.

10.3.6.5 Applications

The pH meter with both a pH scale and millivolt scale may be used for ion-selective electrode analyses as described previously. One simply replaces the glass electrode for measuring pH with the ISE of choice and follows instructions for the determination.

Some examples of applications of ion-selective electrodes are salt and nitrate in processed meats, salt content of butter and cheese, calcium in milk, sodium in low-sodium ice cream, carbon dioxide in soft drinks, potassium and sodium levels in wine, and nitrate in canned vegetables. An ISE method applicable to foods containing <100 mg sodium/100 g is an official method of AOAC International (AOAC Method 976.25). This method employs a sodium combination ISE, pH meter, magnetic stirrer, and a special type of graph paper for plotting a standard curve. Obviously, there are many other applications, but the above serve to demonstrate the versatility of this valuable measuring tool.

A major *advantage* in the use of ion-selective electrodes lies in the ability to measure many anions and cations directly. Such measurements are relatively simple compared to most other analytical techniques, particularly because the pH meter may be used as the voltmeter. Analyses are independent of sample volume when making direct measurements, while turbidity, color, and viscosity are all of no concern.

A major *disadvantage* in the use of ion-selective

10-7 figure Examples of ion-selective electrode calibration curves for ions important in foods. (Courtesy of Phoenix Electrode Co., Houston, TX.)

tial occurs as titrant is added because it is reacting with the sample species. However, when all of the sample species has reacted with the titrant, a large increase in the electrode potential occurs, revealing the equivalence point of the titration (Fig. 10-8).

Finally, the method of standard addition will be considered, as it does have application in ISE methodology. The electrodes (indicator and reference) are immersed in the sample, and the initial electrode

electrodes is their inability to measure below 2–3 ppm, although there are some electrodes that are sensitive down to 1 part per billion. At low levels of measurement (below 10^{-4} M), the electrode response time is slow. Finally some electrodes have had a high rate of premature failure or a short operating life and possible excessive noise characteristics.

10.4 COMPARISON OF METHODS

All minerals of concern nutritionally, for food processing, and toxicologically cannot be assessed by any single method with an equal degree of analytical accuracy. For labeling, processing, and even practical nutrition, we are concerned only with a few minerals, which generally can be analyzed by traditional methods. The traditional methods available for mineral analysis are varied; a very limited number of examples have been given.

Generally, for a small laboratory with skilled analytical personnel, the traditional methods can be carried out rapidly, with accuracy and at minimal costs. If a large number of samples of a specific element are to be run there is certainly a time factor in favor of using atomic absorption spectroscopy or emission spectroscopy (see Chapter 28), depending on the mineral being analyzed. The graphite furnace on the atomic absorption spectrophotometer is capable of sensitivity in the parts-per-billion range. This is beyond the limits of the traditional methods. However, for most minerals of practical concern in the food industry, this degree of sensitivity is not required.

Individual choice of methods for mineral analysis must be made based on cost per analysis completed. Equipment availability, equipment cost, analytical time, analytical volume, and requirements for sensitivity should all be considered in making the final decision on which methods to use.

10.5 SPECIAL CONSIDERATIONS

The Nutrition Labeling and Education Act (NLEA) of 1990 has made health claims on food labels legal under some conditions. Two minerals that are specifically identified as relating to health claims are calcium and sodium. Sodium analysis also is important in making claims for low-sodium-content food items that are being promoted for people with hypertension. Implementation of the NLEA has led to a need for more rapid and accurate analysis of these elements. Traditional methods described in this chapter can be used for quality assurance work and labeling compliance by companies with few specialized products. Methods such as atomic absorption spectrometry and emission spec-

trometry described in Chapter 28 are utilized commonly by laboratories specializing in providing large quantities of mineral data for labeling purposes and for compliance checks.

10.6 SUMMARY

The mineral content of water and foodstuffs is important because of nutritional value, toxicological potential, and proper processing function and texture of some foods. Traditional methods for mineral analysis include gravimetric, titrimetric, and colorimetric procedures. Foods are typically ashed prior to these analyses, since the methods generally require that the minerals be freed from the organic matrix of the foods. Sample preparation must include steps necessary to prevent contamination or loss of volatile elements and must deal with any potential interferences. The basic principles of gravimetric, titrimetric, colorimetric, and ion-selective electrode methods for mineral analyses are described in this chapter, with procedures given for some minerals of concern in the food industry.

The procedures described in this chapter for mineral analyses generally require chemicals and equipment routinely available in an analytical laboratory and do not require expensive instrumentation. These methods may be suited to a small laboratory with skilled analytical personnel and a limited number of samples to be analyzed. Adequate quantities of samples must be available, and a high degree of sensitivity must not be required.

Traditional methods for mineral analysis are being kit-adapted for rapid analysis. Tests for water hardness and the Quantab for salt determination are examples currently being used. The basic principles involved in these methods will continue to be utilized to develop inexpensive rapid methods for screening mineral content of foods and beverages.

Ion-selective electrodes (ISEs) are available for the direct measurement of various cations and anions, such as sodium, potassium, and calcium. It also is possible to measure dissolved gases such as ammonia and carbon dioxide. Since the pH meter has a millivolt scale, it may be used for such measurements by simply replacing the glass electrode with the desired ISE.

10.7 STUDY QUESTIONS

1. What is the major concern in sample preparation for specific mineral analysis? How can this concern be addressed?

2. Calcium can be quantitated by gravimetric analysis, EDTA complexometric titration, and redox titration. Differentiate these techniques with regard to the principles involved.

3. The Mohr and Volhard titration methods often are used to determine the NaCl content of foods. Compare and contrast these two methods, as you explain the principles involved.

4. In a back-titration procedure, would overshooting the endpoint in the titration cause an over- or underestimation of the compound being quantitated? Explain your answer.

5. What is the function of a sample matrix standard? How is it prepared?

6. Describe analytical conditions that may call for the use of a reagent blank.

7. Explain the principles of using an ion-selective electrode to measure the concentration of a particular inorganic element in food. Explain how an ion-selective electrode works and why electrode potential can be correlated to concentration when one is really measuring activity and not concentration.

8. Your lab technician forgot to add the appropriate ionic strength adjustor (ISA) solution to samples and standards when preparing solutions for analysis with an ion-selective electrode. Should you tell the technician to proceed with the samples as already prepared, or go back and prepare samples and standards with ISA solution? Explain your answer, with reference to the principles of using an ISE to quantitate ions.

9. To measure accurately the concentration of a particular element with an ion-selective electrode, ionic strength of the sample being analyzed is only one of the factors that must be controlled. List the other things one must do (i.e., factors to control, consider, or eliminate) for an accurate measure of concentration by the ISE method.

10. You have decided to purchase an ion-selective electrode to monitor the sodium content of foods produced by your plant. List the advantages this would have over the atomic absorption/emission method or the Mohr/Volhard titration method. List the problems and disadvantages of ISE that you should anticipate.

11. What factors should be considered in selecting a specific method for mineral analysis for a food product?

10.8 PRACTICE PROBLEMS

1. If a given sample of food yields 0.750 g of silver chloride in a gravimetric analysis, what weight of chloride is present?

2. A 10 g food sample was dried, then ashed, and analyzed for salt (NaCl) content by the Mohr titration method ($AgNO_3 + Cl^- \rightarrow AgCl$). The weight of the dried sample was 2 g, and the ashed sample weight was 0.5 g. The entire ashed sample was titrated using a standardized $AgNO_3$ solution. It took 6.5 ml of the $AgNO_3$ solution to reach the endpoint, as indicated by the red color of Ag_2CO_4 when K_2CrO_4 was used as an indicator. The $AgNO_3$ solution was standardized using 300 mg of dried KCl as described in Fig. 10-3. The corrected volume of $AgNO_3$ solution used in the titration was 40.9 ml. Calculate the salt (NaCl) content of the original food sample in terms of percent (wt/wt) NaCl.

3. A 25-g food sample was dried, then ashed, and finally analyzed for salt (NaCl) content by the Volhard titration method. The weight of the dried sample was 5 g, and the ashed sample weighed 1 g. Then 30 ml of 0.1 N $AgNO_3$ was added to the ashed sample, the resultant precipitate was filtered out, and a small amount of ferric ammonium sulfate was added to the filtrate. The filtrate was then titrated with 3 ml of 0.1 M KSCN to a red endpoint.
   a. What was the moisture content of the sample, expressed as percent H_2O (wt/wt)?
   b. What was the ash content of the sample, expressed as percent ash (wt/wt) on a dry-weight basis?
   c. What was the salt content of the original sample in terms of percent (wt/wt) NaCl? (molecular weight Na = 23; molecular weight Cl = 35.5)

4. Compound X in a food sample was quantitated by a colorimetric assay. Use the following information and Beer's law to calculate the content of Compound X in the food sample, in terms of mg Compound X/100 g sample:
   a. A 4-g sample was ashed.
   b. Ashed sample was dissolved with 1 ml of acid and the volume brought to 250 ml.
   c. A 0.75-ml aliquot was used in a reaction in which the total volume of the sample to be read in the spectrophotometer was 50 ml.
   d. Absorbance at 595 nm for the sample was 0.543.
   e. The absorptivity constant for the reaction (i.e., molar extinction coefficient) was known to be 1734 liters $gm^{-2} cm^{-1}$.
   f. Inside diameter of cuvette for spectrophotometer was 1 cm.

5. Colorimetric analysis
   a. You are using a colorimetric method to determine the concentration of Compound A in your liquid food sample. This method allows a sample volume of 5 ml. This volume must be held constant but can be comprised of diluted standard solution and water. For this standard curve, you need standards that contain 0, 0.25, 0.50, 0.75, and 1.0 mg of Compound A. Your stock standard solution contains 5 g/liter of Compound A.

      Devise a dilution scheme(s) for preparing the samples for this standard curve that could be followed by a lab technician. Be specific. In preparing the dilution scheme, use no volumes less than 0.2 ml.
   b. You obtain the following absorbance values for your standard curve:

SAMPLE	ABS (500 nm)
0.00 mg	0.00
0.25 mg	0.20
0.50 mg	0.40
0.75 mg	0.60
1.00 mg	0.80

On a sheet of graph paper, construct a standard curve and determine the equation of the line.
   c. A 5-ml sample is diluted to 500 ml, and 3 ml of this solution is analyzed as per the standard samples; the absorbance of 0.50 units at 500 nm. Use the equation of the line calculated in part (b) and information about the dilutions to calculate what the concentra-

tion is of Compound A in your original sample in terms of g/liter.

Answers

1.

$$\frac{x \text{ g Cl}}{0.750 \text{ g AgCl}} = \frac{35.45 \text{ g/mole}}{143.3 \text{ g/mole}}$$

$$x = 0.1855 \text{ g Cl}$$

2.

$$N \text{ AgNO}_3 = \frac{0.3 \text{ g KCl}}{\text{ml AgNO}_3 \times 74.555 \text{ g KCl/mole}}$$

$$0.0984 \, N = \frac{0.3 \text{ g}}{40.9 \text{ ml} \times 74.555}$$

$(0.0984 \, M \text{ AgNO}_3)(0.0065 \text{ liter}) = 0.0006396 \text{ mole Ag}^+$
$\to 0.0006396 \text{ mole Cl}^-$
$\Rightarrow 0.006396 \text{ mole NaCl}$

$(0.0006396 \text{ mole NaCl}) \times \dfrac{58.5 \text{ g NaCl}}{\text{mole}} = 0.0374 \text{ g NaCl}$

3.

a. $\dfrac{25 \text{ g wet sample} - 5 \text{ g dry sample}}{25 \text{ g wet sample}} \times 100 = 80\%$

b. $\dfrac{1 \text{ g ash}}{5 \text{ g dry sample}} \times 100 = 20\%$

c. moles Ag added = moles Cl⁻ in sample + moles SCN⁻ added

moles Ag = (0.1 mole/liter) × (0.03 liter) = 0.003 mole
moles SCN⁻ = (0.1 mole/liter) × (0.003 liter) − 0.0003 mole
0.003 mole Ag = moles Cl⁻ + 0.0003 mole SCN⁻
0.0027 mole = moles Cl⁻

$(0.0027 \text{ mole Cl}^-) \times \dfrac{58.5 \text{ g NaCl}}{\text{mole}} = 0.1580 \text{ g NaCl}$

$\dfrac{0.1580 \text{ g NaCl}}{25 \text{ g wet sample}} = \dfrac{0.00632 \text{ g NaCl}}{\text{g wet sample}} \times 100$

$= 0.632\% \text{ NaCl (wt/wt)}$

4. $A = abc$
0.543 = (1.574 liter g⁻¹ cm⁻¹) (1 cm) c
$c = 3.4498 \times 10^{-4}$ g/liter
$c = 3.4498 \times 10^{-4}$ mg/ml

$\dfrac{3.4498 \times 10^{-4} \text{ mg}}{\text{ml}} \times 50 \text{ ml} = 1.725 \times 10^{-2} \text{ mg}$

$\dfrac{1.725 \times 10^{-2} \text{ mg}}{0.75 \text{ ml}} \times \dfrac{250 \text{ ml}}{4 \text{ g}} = 1.437 \text{ mg/g}$

$= 143.7 \text{ mg/100 g}$

5. a. Say that you want to know what dilution to do on the 5 mg/ml stock solution to pipette 0.2 ml for the lowest point on the standard curve (0.25 mg/5 ml). What dilution must you do?

$\dfrac{0.25 \text{ mg}}{5 \text{ ml}} \times \dfrac{5 \text{ ml}}{0.2 \text{ ml}} \times \dfrac{x \text{ ml}}{1 \text{ ml}} = \dfrac{5 \text{ mg}}{\text{ml}}$

$$x = 4 \text{ ml}$$

∴ Dilution of 1 ml to 4 ml would give 0.25 mg in a 0.2-ml sample.
Say that you have diluted the 5 mg/ml solution by 1 to 10. How much of this diluted solution do you pipette to get a concentration of 0.25 mg/5 ml (in the total sample volume)?

$\dfrac{0.25 \text{ mg}}{5 \text{ ml}} \times \dfrac{5 \text{ ml}}{x \text{ ml}} \times \dfrac{10 \text{ ml}}{1 \text{ ml}} = \dfrac{5 \text{ mg}}{\text{ml}}$

$$x = 0.5 \text{ ml}$$

∴ Dilution of 1 ml to 10 ml would give 0.25 mg in a 0.5-ml sample.
Using diluted stock solution of 0.25 mg/0.5 ml:

$\dfrac{0.5 \text{ mg}}{\text{ml}} \times x \text{ ml} = 1.0 \text{ mg}$

$$x = 2 \text{ ml}$$

etc. for 0.75, 0.50, 0.25 mg

mg A/5 ml	ml diluted stock solution	ml H₂O
0	0	5.0
.25	5	4.5
.50	1.0	4.0
.75	1.5	3.5
1.0	2.0	3.0

b.
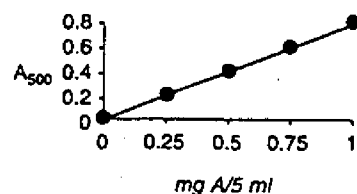
mg A/5 ml
Equation of the line: y = 0.8x + 0

c.
$A_{500} = 0.50 = y$
$0.50 = 0.8x + 0$
$x = 0.625$

$\dfrac{0.625 \text{ mg}}{5 \text{ ml}} \times \dfrac{5 \text{ ml}}{3 \text{ ml}} \times \dfrac{500 \text{ ml}}{5 \text{ ml}} = 20.83 \text{ mg/ml}$

$= 20.83 \text{ g/liter}$

10.9 REFERENCES

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ACKNOWLEDGMENT

11 chapter

Carbohydrate Analysis

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11.1 INTRODUCTION

Carbohydrates are important in foods as a major source of energy, as imparters of crucial physical properties, and as modifiers of human physiological processes. Worldwide, carbohydrates account for > 70% of the caloric value of the human diet. It is recommended that all individuals should limit the calories from fat (the other significant source) to not more than 30% and that most of the carbohydrate calories should come from starch. Carbohydrates provide to foods many attributes, including bulk, body, viscosity, stability to emulsions and foams, water-holding capacity, freeze–thaw stability, browning, flavors, aromas, and a range of desirable textures (from crispiness to smooth, soft gels). Carbohydrates also provide satiety. Basic carbohydrate structures, chemistry, and terminology can be found in references (1) and (2).

Primary occurrences of major carbohydrates in foods are presented in Table 11-1. Carbohydrates are almost exclusively of plant origin, with milk lactose being the major exception. Of the monosaccharides (sometimes called simple sugars), only D-glucose and D-fructose are found in other than minor amounts. These and other monosaccharides are the only carbohydrates that can be absorbed from the small intestine. Higher saccharides (oligo- and polysaccharides) must first be digested, i.e., hydrolyzed to monosaccharides, before absorption and utilization can occur. Humans can digest only sucrose, lactose, maltooligosaccharides, and starch. All are digested with enzymes of the small intestine.

At least 90% of the carbohydrate in nature is in the form of polysaccharides. As stated, starch polymers are the only polysaccharides that humans can digest and use as a source of calories. Other polysaccharides are nondigestible. Nondigestible polysaccharides can be divided into soluble and insoluble classes and, along with lignin, make up dietary fiber (see Chapter 12). As soluble and insoluble dietary fiber, they regulate normal bowel function, reduce the hyperglycemic response, and may lower serum cholesterol. However, nondigestible polysaccharides most often are added to processed foods because of the functional properties they impart, rather than for their physiological effect. Nondigestible oligosaccharides serve as prebiotics and are, therefore, increasingly common ingredients in functional foods and neutraceuticals.

Carbohydrate analysis is important from several perspectives. Qualitative analysis ensures that ingredient labels present accurate compositional information. Quantitative analysis ensures that added components are listed in proper order on the ingredient label. Quantitative analysis also ensures that labeled amounts of specific components of consumer interest, for example, β-glucan, are proper. Both qualitative and quanti-

tative analysis can be used to detect adulteration of products such as fruit juices.

In this chapter, the most commonly used methods of carbohydrate determination are presented. However, particular methods often must be made specific to a particular food product because of the nature of the product and the presence of other constituents. Approved methods are at least referenced, but methods approval has not kept pace with methods development; so where better methods are available, they are presented. Methods that have been in long-time use, although not giving as much or as precise information as newer methods, nevertheless can be useful for quality assurance and product standardization. References (3) and (4) contain additional information on determination of food carbohydrates.

In general, evolution of analytical methods for carbohydrates has followed the succession: qualitative color tests, adaptation of the color test for reducing sugars based on reduction of Cu^{2+} to Cu^+ (Fehling test) to quantitation of reducing sugars, qualitative paper chromatography, quantitative paper chromatography, gas chromatography of derivatized sugars, qualitative and quantitative thin-layer chromatography, enzymatic methods, high performance liquid chromatography. Methods employing nuclear magnetic resonance (Chapter 30), mass spectrometry (Chapter 29), capillary electrophoresis, near-infrared (NIR) spectrometry (section 11.6.2), antibodies (immunoassays, Chapter 21), and fluorescence spectrometry (Chapter 26) have been published, but are not yet in general use for carbohydrate analysis. Methods continue to evolve and be developed.

It should be noted that, according to nutrition labeling regulations of the United States Food and Drug Administration, the "total carbohydrate" content of a food (Table 11-2) must be calculated by subtraction of the sums of the weights of crude protein, total fat, moisture, and ash from the total weight of the food (5), i.e., carbohydrate is determined by difference. The content of "other carbohydrate" (formerly called "complex carbohydrate") is obtained by calculating the difference between the amount of "total carbohydrate" and the sum of the amounts of dietary fiber, sugars, and sugar alcohol (Table 11-1), where sugars are defined as glucose, fructose, sucrose, and lactose and the sugar alcohol is sorbitol (5).

11.2 SAMPLE PREPARATION

Sample preparation is related to the specific raw material, ingredient, or food product being analyzed and the specific carbohydrate being determined, because carbohydrates have such a wide range of solubilities. However, some generalities can be presented (Fig. 11-1).

11-1
table Some Occurrences of Major Carbohydrates In Foods

Carbohydrate	Source.	Constituent(s)
Monosaccharides[1]		
D-Glucose (Dextrose)	Naturally occurring in honey, fruits, and fruit juices. Added as a component of corn (glucose) syrups and high-fructose corn syrup. Produced during processing by hydrolysis (inversion) of sucrose.	
D-Fructose	Naturally occurring in honey, fruits, and fruit juices. Added as a component of high-fructose corn syrup. Produced during processing by hydrolysis (inversion) of sucrose.	
Sugar alcohol[1]		
Sorbitol (D-Glucitol)	Added to food products, primarily as a humectant.	
Disaccharides[1]		
Sucrose	Widely distributed in fruit and vegetable tissues and juices in varying amounts. Added sugar (crystalline and liquid)	D-Glucose D-Fructose
Lactose	In milk and products derived from milk	D-Galactose D-Glucose
Maltose	In malt. In varying amounts in various corn (glucose) syrups and maltodextrins	D-Glucose
Higher oligosaccharides[1]		
Maltooligosaccharides	In varying amounts in various corn (glucose) syrups and maltodextrins	D-Glucose
Raffinose	Small amounts in beans	D-Glucose D-Fructose D-Galactose
Stachyose	Small amounts in beans	D-Glucose D-Fructose D-Galactose
Polysaccharides		
Starch[2]	Widespread in cereal grains and tubers. Added to processed foods.	D-Glucose
Food gums/hydrocolloids[3] Algins Carboxymethylcelluloses Carrageenans Guar gum Gum arabic Hydroxypropylmethylcelluloses Locust bean gum Methylcelluloses Pectins Xanthan	Added as ingredients	([4])
Cell-wall polysaccharides[3] Pectin (native) Cellulose Hemicelluloses β-Glucan	Naturally occurring	

[1]For analysis, see section 11.3.
[2]For analysis, see section 11.4.1.1.
[3]For analysis, see section 11.4.2.
[4]For compositions, characteristics, and applications, see reference (2) (Table 11-2).

11-2 table	Total Carbohydrate Contents of Selected Foods	

Food	Appropriate Percent Carbohydrate (wet weight basis)
Cereals, bread, and pasta	
Corn flakes	86
Granola bars, low fat	79–82
Granola bars	71–75
Macaroni, dry, enriched	75
Bread, white	50
Dairy products	
Ice cream	22–27
Yogurt, plain	4.7–6.9
Milk, whole	4.7
Fruits and vegetables	
Applesauce, canned, sweetened	20
Grapes	16–17
Apples, raw, with skin	15
Potatoes, raw, with skin	12
Orange juice	10–11
Carrots, raw	10
Broccoli, raw	5.2
Tomato, tomato juice	4.2
Meat, poultry, and fish	
Fish fillets, battered or breaded	17–19
Bologna and other luncheon meats	4
Chicken, broilers or fryers, breast meat	0
Other	
Honey	75–82
Milk chocolate	59
Salad dressing, pourable, fat-free	10–34
Salad dressing, pourable	3.3–22
Soft drinks, caloric	11–12
Iced tea, sweetened, bottled	7.1–11
Cream of mushroom soup, from condensed and canned	7.4
Light beer	1.3

In part from USDA Nutrient Database for Standard Reference Release 11-1 (August 1997)
http://www.nal.usda.gov/fric/cgi-bin/nut_search.pl

For most foods, the first step is drying, which also can be used to determine moisture content. For other than beverages, drying is done by placing a weighed amount of material in a vacuum oven and drying to constant weight at 55°C and 1 mm Hg pressure. Then, the material is ground to a fine powder, and lipids are extracted using 95:5 vol/vol chloroform–methanol in a Soxhlet extractor (see Chapter 13). Prior extraction of lipids makes extraction of carbohydrates easier and more complete.

However, other sample preparation schemes may be required. For example, the AOAC International method (6) for presweetened, ready-to-eat breakfast cereals calls for removal of fats by extraction with petroleum ether (hexane) and extraction of sugars with

Raw material,
Ingredient, or
Finished product

Dry

Water   Dehydrated material

1. Grind
2. 95:5 CHCl₃-MeOH

Lipids and lipid-soluble components   Residue

80% ethanol

Ion exchange   Residue

Mono- and disaccharides
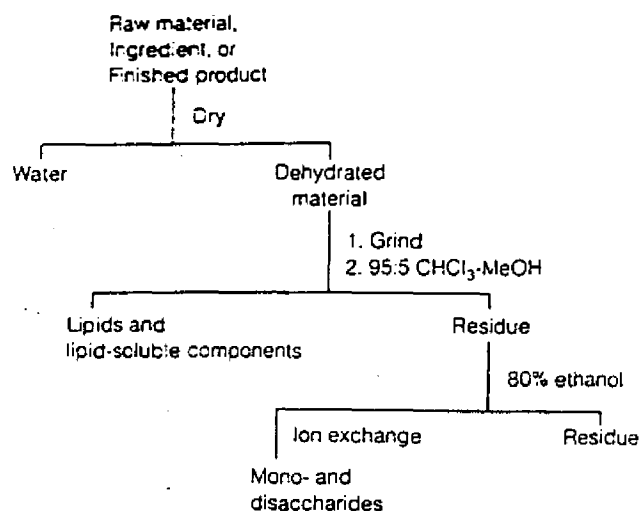
11-1 figure	General scheme for sample preparation and extraction of mono- and disaccharides.

50% ethanol (AOAC method 982.14), rather than the method described below.

11.3 MONO- AND OLIGOSACCHARIDES

11.3.1 Extraction

Foodstuffs and food products are complex, heterogeneous, biological materials. Thus, it is quite likely that they may contain substances that interfere with measurement of the mono- and oligosaccharides present, especially if a spectrophotometric method is used. Interference may arise either from compounds that absorb light of the same wavelength used for carbohydrate analysis or from insoluble, colloidal material that scatters light. (Light scattering is measured as absorbance.) Also, the aldehyde or ketone group of the sugar can react with other components, especially amino groups of proteins, a reaction (the nonenzymatic browning or Maillard reaction) that both produces color and destroys the sugar that needs to be measured. Even if chromatographic methods, such as HPLC (section 11.3.4.1), are used for analysis, substances that might ruin the column or other components of the system must be removed first. Thus, for determination of any mono- (glucose, fructose), di- (sucrose, lactose, maltose), tri- (raffinose), tetra- (stachyose), or other oligo- (maltodextrins) saccharides present, the dried, lipid-free sample is extracted with hot 80% ethanol (final concentration, in the presence of precipitated calcium carbonate to neutralize any acidity) (AOAC Method 922.02, 925.05). Higher oligosaccharides from added malto- or fructooligosaccharides also may be extracted. Carbohydrates are soluble in polar solvents.

However, much of the composition of a food (other than water) is in the form of polymers, and almost all polysaccharides and proteins are insoluble in hot 80% ethanol. Thus, this extraction is rather specific. This extraction is done by a batch process. Refluxing for 1 hr, cooling, and filtering is standard. (A Soxhlet apparatus with the sample in a thimble in the middle section of the extraction unit cannot be used because aqueous ethanol undergoes azeotropic distillation as 95% ethanol.) Extraction should be done at least twice to check for and ensure completeness of extraction. If the foodstuff or food product is particularly acidic, for example, a low-pH fruit, neutralization before extraction may be necessary to prevent hydrolysis of sucrose, which is particularly acid labile; thus, precipitated calcium carbonate is added routinely.

The 80% ethanol extract will contain components other than carbohydrates, in particular ash, pigments, organic acids, and perhaps free amino acids and low-molecular-weight peptides. Because the mono- and oligosaccharides are neutral and the contaminants are charged, the contaminants can be removed by ion-exchange techniques (see Chapter 31). Because reducing sugars can be absorbed on and isomerized by strong anion-exchange resins in the hydroxide form, a weak anion-exchange resin in the carbonate ($CO^{2-}{}_3$) or hydrogencarbonate ($HCO^-{}_3$) form should be used. [Reducing sugars are those mono- and oligosaccharides that contain a free carbonyl (aldehyde or ketone) group and, therefore, can act as reducing agents; see section 11.3.3.] Because sucrose and sucrose-related oligosaccharides are very susceptible to acid-catalyzed hydrolysis, the anion-exchange resin should be used before the cation-exchange resin. However, because the anion-exchange resin is in a carbonate or hydrogencarbonate form, the cation-exchange resin (in H^+ form) cannot be used in a column because of CO_2 generation. Mixed-bed columns are not recommended for the same reason. AOAC Method 931.02C reads basically as follows for clean-up of ethanol extracts: Place a 50-ml aliquot of the ethanol extract in a 250-ml Erlenmeyer flask. Add 2 g of cation-exchange resin (acid form) and 3 g of anion-exchange resin (hydroxide form) (AOAC Method 931.02C). Let stand 2 hr with occasional swirling.

The classic method for determining sucrose concentration by polarimetry (section 11.6.5) requires a clear solution, and thus application of a clarifying agent. In place of an ion-exchange treatment, addition of basic lead acetate or an alternative reagent, followed by filtration or centrifugation, is recommended (AOAC Methods 44.1.07B, 44.2.10, 44.6.01).

The aqueous alcohol of the ethanol extract is removed under reduced pressure using a rotary evaporator and a temperature of 45–50°C. The residue is dissolved in a known, measured amount of water. Filtra-

tion should not be required, but should be used if necessary. Some methods employ a final passage through a hydrophobic column (Sep-Pak C18 cartridge, Waters Associates, Milford, MA) as a final clean-up step to remove any residual lipids, proteins, or pigments, but this should not be necessary if the lipids and lipid-soluble components were properly removed prior to extraction. (Extracts may contain minor carbohydrates, such as cyclitols and naturally occurring or added alditols. These are not considered in sections 11.3.2 or 11.3.4.)

11.3.2 Total Carbohydrate: Phenol–Sulfuric Acid Method

11.3.2.1 Principle and Characteristics

Carbohydrates are destroyed by heat and acid. They are particularly sensitive to strong acids and high temperatures. Under these conditions, a series of complex reactions take place, beginning with a simple dehydration reaction as shown in Equation [1]:

$$-\underset{\underset{OH}{|}}{\overset{\overset{H}{|}}{C}}-\underset{\underset{OH}{|}}{\overset{\overset{H}{|}}{C}}- \quad \longrightarrow \quad -\underset{\underset{OH}{|}}{\overset{\overset{H}{|}}{C}}=C- \quad \rightleftharpoons \quad -CH_2-\underset{\overset{||}{O}}{C}- \quad [1]$$
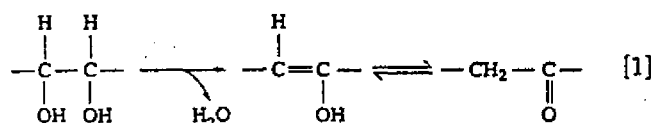
Continued heating in the presence of acid produces various furan derivatives (Fig. 11-2). These products then condense with themselves and other products to produce brown and black substances. They will also condense with various phenolic compounds, such as phenol, resorcinol, orcinol, α-naphthol, and napthoresorcinol, and with various nitrogen-containing compounds, particularly nitrogen heterocycles, to produce colored compounds that are useful for carbohydrate analysis (3).

The most widely used condensation is with phenol itself (4, 7-9, AOAC Method 44.1.30). This method is simple, rapid, sensitive, accurate, specific for carbohydrates, and widely applied. Virtually all classes of sugars, including sugar derivatives and oligo- and polysaccharides, can be determined. (Oligo- and polysaccharides react because they undergo hydrolysis in the presence of the hot, strong acid, releasing monosaccharides.) The reagents are inexpensive, readily available, and stable. A stable color is produced, and results are reproducible. Under proper conditions, the phenol–sulfuric method is accurate to ±2%.

With this method and with methods for measuring reducing sugar content (section 11.3.3), the response is never stoichiometric and is, in part, a function of the structure of the sugar. Therefore, a standard curve must be used. Ideally, the standard curve will be prepared using mixtures of the sugars present in the same ratio as they are found in the unknown. If this is not

Furaldehyde
(Furfural)

Hydroxymethylfuraldehyde
(HMF)

11-2
figure

Furan products that could arise from, in order, pentoses and hexuronic acids, hexoses, 6-deoxyhexoses, and keto-hexoses [see reference (1)].

possible, for example, if a pure preparation of the sugar being measured is not available, or if more than one sugar is present either as free sugars in unknown proportions or as constituent units of oligo- or polysaccharides or mixtures of them, D-glucose is used to prepare the standard curve. In these cases, accuracy is determined by conformity of the standard curve made with D-glucose to the curve that would be produced from the exact mixture of pure carbohydrates being determined. In any analysis, the concentrations used to construct the standard curve must span the sample concentrations and beyond, i.e., all sample concentrations must fall within the limits of the standard concentrations, and both must be within the limits reported for sensitivity of the method. If any concentrations are greater than the upper limit of the sensitivity range, dilutions can, and should, be used.

11.3.2.2 Outline of Procedure

1. A clear, aqueous solution of carbohydrate(s) is pipetted into a small tube. A blank of water also is prepared.
2. An aqueous solution of phenol is added, and the contents are mixed.
3. Concentrated sulfuric acid is added rapidly to the tube so that the stream produces good mixing. The tube then is agitated. (Adding the sulfuric acid to the water produces considerable heat.) A yellow-orange color results.
4. Absorbance is measured at 490 nm.
5. The average absorbance of the blanks is subtracted, and the amount of sugar is determined by reference to a standard curve (section 11.3.2.1).

11.3.3 Total Reducing Sugar

11.3.3.1 Somogyi–Nelson Method

11.3.3.1.1 Principle The most often used method to determine amounts of reducing sugars is the Somogyi–Nelson method (4, 9–12). This and other reducing sugar methods (section 11.3.3.2) can be used in combi-

nation with enzymatic methods (section 11.3.4.3) for oligo- and polysaccharide determination. In these cases, specific hydrolases are used to convert the oligo- or polysaccharide into its constituent monosaccharide or repeating oligosaccharide units which are measured using a reducing sugar method.

$$R\!-\!\overset{\overset{\displaystyle O}{\|}}{C}\!-\!H + 2\,Cu(OH)_2 \longrightarrow R\!-\!\overset{\overset{\displaystyle O}{\|}}{C}\!-\!O^-Na^+ \quad\quad [2]$$
$$+ \; NaOH \quad\quad\quad\quad + \; Cu_2O + 3\,H_2O$$

The Somogyi–Nelson method is based on reduction of Cu^{2+} ions to Cu^+ ions by reducing sugars. The Cu^+ ions then reduce an arsenomolybdate complex, which is prepared by reacting ammonium molybdate [$(NH_4)_6Mo_7O_{24}$] and sodium arsenate (Na_2HAsO_7) in sulfuric acid. Reduction of the arsenomolybdate complex produces an intense, stable, blue color that is measured spectrophotometrically. This reaction is not stoichiometric and must be used with a standard curve of the sugar(s) being determined or with D-glucose.

11.3.3.1.2 Outline of Procedure

1. A solution of copper(II) sulfate and an alkaline buffer are added by pipets to a solution of reducing sugars(s) and a water blank.
2. The resulting solution is heated in a boiling water bath.
3. A reagent prepared by mixing solutions of acidic ammonium molybdate and sodium arsenate is added.
4. After mixing, dilution, and remixing, absorbance is measured at 520 nm.
5. After subtraction of the absorbance of the reagent blank, the A_{520} is converted into glucose equivalents using a standard plot of micrograms of glucose versus absorbance (section 11.3.2.1).

11.3.3.2 Other Methods

The dinitrosalicylic acid method (13) also may be used to measure reducing sugars naturally occurring in foods or released by enzymes. In this reaction, 3,5-dini-

trosalicylate is reduced to the reddish monoamine derivative:

$$HC=O$$

HCOH

3 HOCH +

HCOH O_2N

HCOH

CH_2OH

D-Glucose

COO^-Na^+

OH

O_2N NO_2

Sodium 3, 5-Dinitrosalicylate

+ 3 OH⁻ ⟶

COO^-

HCOH

3 HOCH +

HCOH O_2N

HCOH

CH_2OH

D-Gluconate

COO^-Na^+

OH

NH_2

Sodium 3-Amino-5-nitrosalicylate

+ 2 H_2O

[3]

Other methods are, like the Somogyi–Nelson method, based on reduction of copper(II) ions in alkaline solution to copper(I) ions that precipitate as the brick-red oxide Cu_2O. Tartrate or citrate ions are added to keep the copper(II) ions in solution under the alkaline conditions.

The Munson–Walker method (AOAC Method 906.03) has various forms. The precipitate of cuprous oxide can be determined gravimetrically (AOAC Method 31.039, 14th ed.), by titration with sodium thiosulfate (AOAC Method 31.040, 14th ed.), by titration with potassium permanganate (AOAC Method 31.042, 14th ed.), by titration in the presence of methylene blue (the Lane–Eynon method; AOAC Method 923.09, 920.183b), and electrolytically (AOAC Method 31.044, 14th ed.) (4). These methods also must be used with standard curves because each reducing sugar reacts differently. Because assay conditions affect the outcome, they generally also must be done by trained, experienced analysts so that they always are done exactly the same way. These methods are still used where specified. Although a ketone group cannot be oxidized to a carboxylic acid group, and thus ketoses are not reducing sugars, under the alkaline conditions employed, ketoses are isomerized to aldoses (1), and thus are measured as reducing sugars. However, the response is less with ketoses, so a different standard curve should be used if D-fructose is present.

Methods that both identify individual carbohydrates present and determine their amounts are preferred over general reducing sugar methods and are described next.

11.3.4 Specific Analysis of Mono- and Oligosaccharides

11.3.4.1 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) (Chapter 32) is the method of choice for analysis of mono- and oligosaccharides and can be used for analysis of polysaccharides after hydrolysis (section 11.4.2). HPLC gives both qualitative analysis (identification of the carbohydrate) and, with peak integration, quantitative analysis. HPLC analysis is rapid, can tolerate a wide range of sample concentrations, and provides a high degree of precision and accuracy. HPLC requires no prior derivatization of carbohydrates, as does gas chromatography (section 11.3.4.2), but does require micron-filter filtration prior to injection. Complex mixtures of mono- and oligosaccharides can be analyzed. The basic principles and important parameters of HPLC (the stationary phase, the mobile phase, and the detector) are presented and discussed in Chapter 32. Some details related to carbohydrate analysis are discussed here. Specific details of methods of analyses of specific food ingredients or products should be obtained from the literature. Use of HPLC to determine soluble food carbohydrates has been tabulated (14).

11.3.4.1.1 Stationary Phases

1. Anion-Exchange Chromatography. Carbohydrates are weak acids and have pK_a values in the range 12–14. In a high pH solution, some of the hydroxyl groups of carbohydrates are ionized, allowing sugars to be separated by anion-exchange resins. Special column packings have been developed for this purpose. The general elution sequence is sugar alcohols (alditols), monosaccharides, disaccharides, and oligosaccharides.

Anion-exchange chromatography is used most often in conjunction with electrochemical detection (see Chapter 32 and section 11.3.4.1.2) Anion-exchange chromatography has been used to examine the complex oligosaccharide patterns of honey (15), brewing syrups (16), beet sugar hydrolysates (17), and orange juice (17). The method has the advantage of being applicable to baseline separation within each class of carbohydrates and of providing separation of homologous series of oligosaccharides into their components (18) (Fig. 11-3).

2. Normal-Phase Chromatography. In normal-
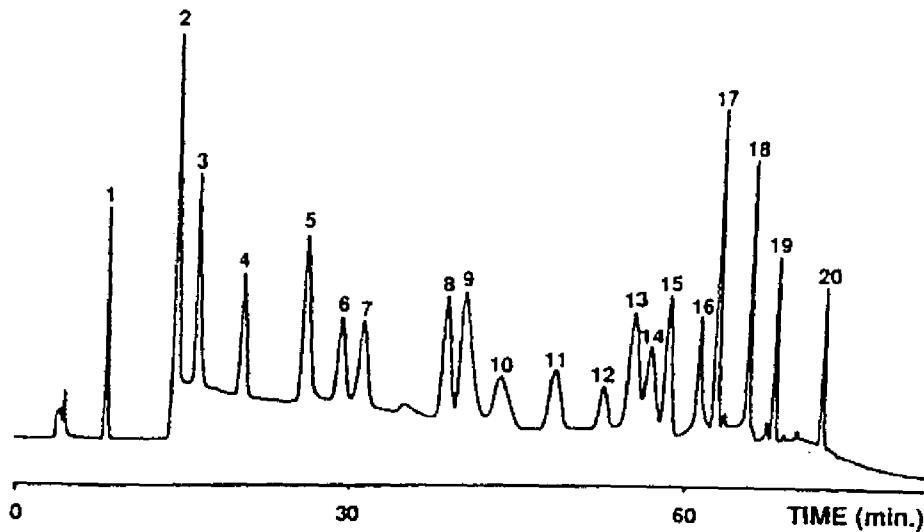
11-3
figure

High performance liquid chromatogram of the mono- and oligosaccharides of honey employing anion-exchange chromatography and pulsed amperometric detection. [From (15), used with permission.] Peak 1, neotrehalose; 2, glucose; 3, fructose; 4, melibiose; 5, isomaltose, maltulose; 6, sucrose; 7, kojibiose; 8, turanose, gentiobiose; 9, platinose; 10, melezitose; 11, isomaltotriose; 12, nigerose; 13, maltose, 1-kestose; 14, theanderose; 15, laminaribiose; 16, isopanose; 17, erlose; 18, panose; 19, maltotriose; 20, laminaritriose.

phase chromatography, the stationary phase is polar and elution is accomplished by employing a mobile phase of increasing polarity. It is a widely used HPLC method for carbohydrate analysis. Silica gel that has been derivatized with one or more of several reagents to incorporate amino groups is used. These so-called amine-bonded stationary phases, that are generally used with acetonitrile–water (50–85% acetonitrile) as the eluent, are effective in carbohydrate separations. The elution order is monosaccharides and sugar alcohols, disaccharides, and oligosaccharides. Amine-bonded silica gel columns have been used successfully to analyze the carbohydrate content of foods such as honey, beverages, breakfast cereals, ice cream, cakes, snacks, infant foods, fruits, vegetables, and meat products (19).

A severe disadvantage of amine-bonded silica gel is the tendency for reducing sugars to react with the amino groups of the stationary phase, which results in a deterioration of column performance over time. This situation can be partially alleviated through the use of amine-modified silica gel columns. To prepare amine-modified silica gel columns, small amounts of modifiers, which are soluble amine compounds, are added to the mobile phase to modify the packing in situ. The modifier must have at least two amino groups, for one is needed to adsorb to the silica gel and the other must be free for the carbohydrate. Because the modifier is in the eluent, the column is continuously regenerated.

3. Cation-Exchange Chromatography. Microparticulate spheres of sulfonated resin are used for cation-exchange stationary phases. The resin is loaded with

one of a variety of metal counter ions, depending on the type of separation desired. Usually Ca^{2+}, Pb^{2+}, or Ag^+ is used as the counter ion. The mobile phase used with these columns is water plus varying amounts (typically < 40%) of an organic solvent such as acetonitrile and/or methanol. These columns normally are operated at elevated temperatures (>80°C) to increase column efficiency by increasing the mass transfer rate between the stationary and mobile phases, resulting in peak narrowing and improved resolution (20).

Carbohydrate elution from cation-exchange resins takes place in order of decreasing molecular weight. Oligosaccharides with a degree of polymerization (DP) greater than 3 elute first, followed by trisaccharides, disaccharides, monosaccharides, and alditols. There is some resolution of disaccharides, but the real strength of this stationary phase is in the separation of individual monosaccharides.

4. Reversed-Phase Chromatography. In reversed-phase chromatography, the stationary phase is hydrophobic, and the mobile phase is largely water (Fig. 11-4). The hydrophobic stationary phase is made by reacting silica gel with a reagent that adds alkyl chains, such as an 18-carbon atom alkyl chain (a C18 column) or a phenyl group (a phenyl column). Reversed-phase chromatography has been used for separation of mono-, di-, and trisaccharides by groups (21), for example, for determination of sucrose, raffinose, and stachyose in soybeans and soy products (22) and for determination of invert sugar, sucrose, maltose, and maltotriose in juices, syrups, and brewery worts (20,23).

A major disadvantage of this stationary phase is
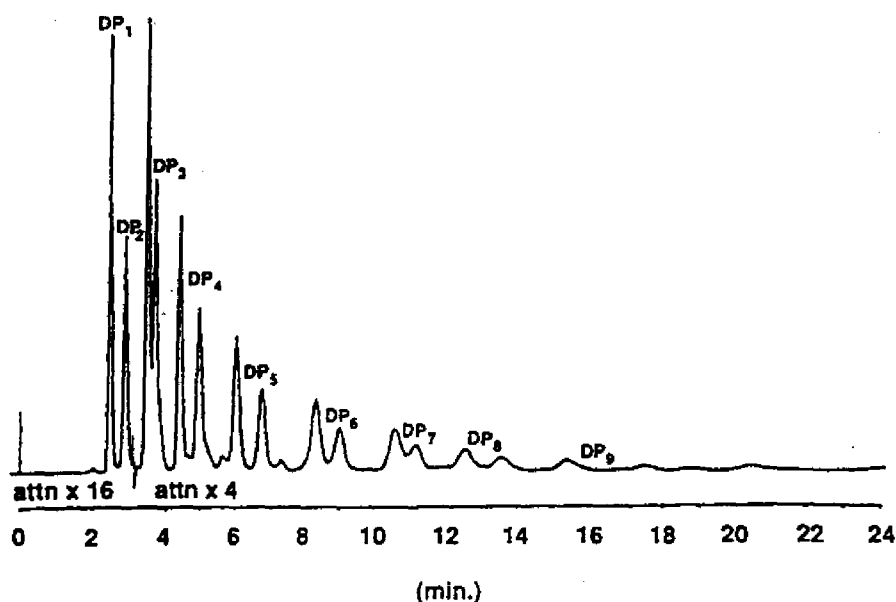
High performance, reversed-phase liquid chromatogram of maltodextrins (DP 1-9). [From (24), used with permission.]

the short retention times of monosaccharides, which result in elution as a single unresolved peak. The addition of salts (such as sodium chloride) can increase retention on the stationary phase and the utility of this method for monosaccharide analysis (25). Reverse-phase chromatography is complicated by peak doubling and/or peak broadening due to the presence of anomers. This problem can be alleviated by the addition of an amine to the mobile phase to accelerate anomerization (mutarotation), but separation may be negatively affected by shorter retention times.

A wide variety of stationary phases is available, including phases not included in one of the four groups given above, and new improved phases continue to be developed. Both normal- and reversed-phase columns have long lives, have good stability over a wide range of solvent composition and pH (from pH 2 to pH 10), are suitable for the separation of a range of carbohydrates, and are of relatively low cost. All silica-based stationary phases share the disadvantage that silica dissolves to a small extent in water-rich eluents.

11.3.4.1.2 Detectors

1. **Refractive Index Detection.** The refractive index (RI) detector is commonly employed for carbohydrate analysis. RI measurements are linear over a wide range of carbohydrate concentrations and can be universally applied to all carbohydrates, but the RI detector has its drawbacks. RI is a bulk physical property that is sensitive to changes in flow, pressure, and temperature, but with modern HPLC equipment and a temperature-controlled detector, problems arising from these changes can be minimized. The most signif-

icant limiting factor with RI detection is that gradient elution cannot be used. The other is that, since an RI detector measures mass, it is not sensitive to low concentrations.

2. **Electrochemical Detection.** The triple-pulsed electrochemical detector, called a pulsed amperometric detector (PAD), which relies on oxidation of carbohydrate hydroxyl and aldehydo groups, is universally used with anion-exchange chromatography (Fig. 11-3) (15). It requires a high pH and, thus, post-column addition of sodium hydroxide solution, which requires an additional pump. It can be used with gradient elutions. The solvents employed are simple and inexpensive (water or sodium hydroxide–sodium acetate solution). Limits are approximately 1.5 ng for monosaccharides and 5 ng for di-, tri-, and tetrasaccharides. The detector is suitable for both reducing and nonreducing carbohydrates; detection limits are slightly lower for reducing sugars.

3. **Post-Column Derivatization** (4). Post-column derivatization involves addition of reagents that will provide colored compounds whose concentration can be measured using absorbance (visible) or fluorescence detection. Post-column reaction is straightforward; requires only one or two additional pumps, a mixing coil, and a thermostatted bath; and provides greater sensitivity than does an RI detector.

11.3.4.2 Gas Chromatography

For gas chromatography (GC) (gas–liquid chromatography, GLC), sugars must be converted into volatile derivatives. The most commonly used derivatives are

the alditol peracetates (and aldonic acid peracetates from uronic acids) (26,27). These derivatives are prepared as illustrated in Fig. 11-5 for D-galactose and D-galacturonic acid. Conversion of sugars into peracetylated aldononitrile (aldoses) and peracetylated ketooxime (ketoses) derivatives for GC is also done (28), although this procedure is not used nearly as much as the preparation of peracetylated aldoses and aldonic acids. However, GC analysis of sugars has been, for the most part, replaced by HPLC. Like HPLC, GC provides both qualitative and quantitative analysis of carbohydrates. A flame ionization detector is the detector of choice for carbohydrates.

The most serious problem with GC for carbohydrate analysis is that two preparation steps are involved: reduction of aldehyde groups to primary alcohol groups, and conversion of the reduced sugar into a volatile peracetate ester or pertrimethylsilyl ether derivative. Of course, for the analysis to be successful, each of these steps must be 100% complete. The basic principles and important parameters of GC (the stationary phase, temperature programming, and detection) are presented and discussed in Chapter 33.

11.3.4.2.1 Neutral Sugars: Outline of Procedure (4,29)

1. Reduction to Alditols. Neutral sugars from the 80% ethanol extract (section 11.3.1) or from hydrolysis

of a polysaccharide (section 11.4.2.2) are reduced with an excess of sodium borohydride dissolved in a solution of ammonium hydroxide. After reaction at room temperature, glacial acetic acid is added dropwise until no more hydrogen is evolved. This treatment destroys excess sodium borohydride. The acidified solution is evaporated to dryness. Borate ions are removed as methyl borate by successive additions and evaporation of methanol.

A potential problem is that, if fructose is present, either as a naturally occurring sugar, from the hydrolysis of inulin, or as an additive (from high fructose corn syrup [HFCS] or invert sugar, for example), it will be reduced to a mixture of D-glucitol (sorbitol) and D-mannitol (Fig. 11-6).

2. Acetylation of Alditols. Acetic anhydride is added. The flask is stoppered and heated at 121°C, then cooled. Water is added to decompose excess acetic anhydride, and the contents are evaporated to dryness.

3. GC of Alditol Peracetates (30). The residue is dissolved in reagent-grade chloroform. Alditol acetates may be chromatographed isothermally and identified by their retention times relative to that of inositol hexacetate added as an internal standard (see section 11.4.2.1). It is wise to run standards of the additol peracetates of the sugars being determined with inositol peracetate as an internal standard.
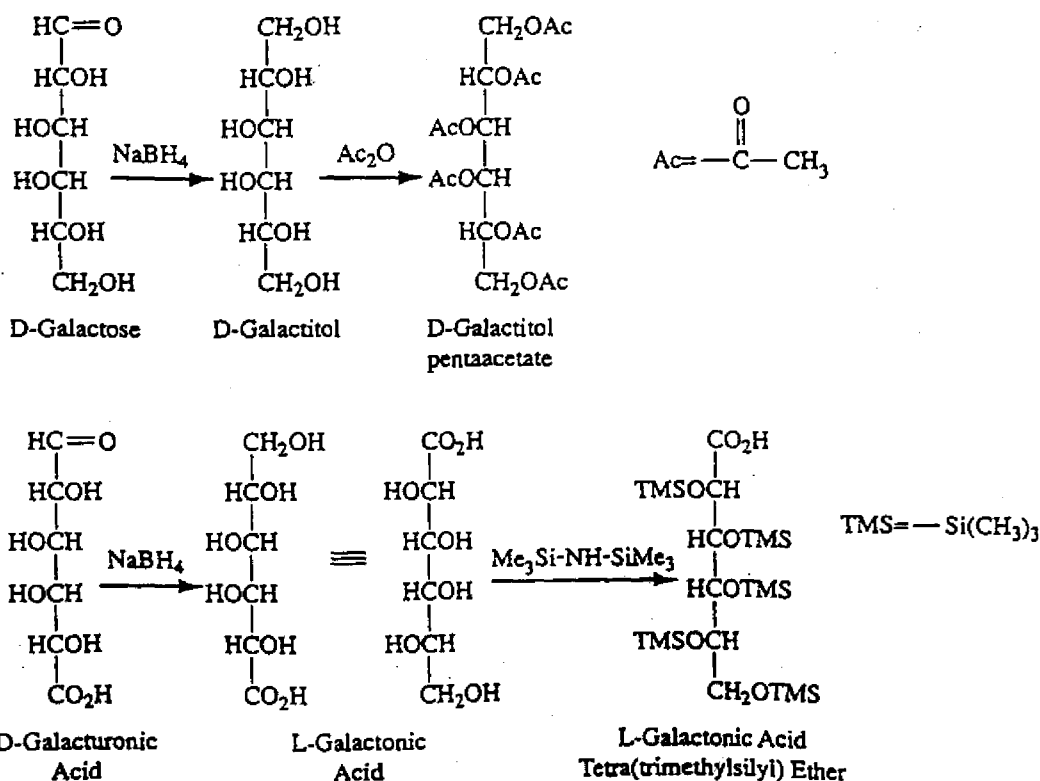
Figure 11-5 Modification of D-galactose and D-galacturonic acid in preparation for gas chromatography.

$$
\begin{array}{ccccc}
\text{CH}_2\text{OH} & & \text{CH}_2\text{OH} & & \text{CH}_2\text{OH} \\
| & & | & & | \\
\text{C}=\text{O} & & \text{HCOH} & & \text{HOCH} \\
| & \xrightarrow{\text{NaBH}_4} & | & + & | \\
\text{HOCH} & & \text{HOCH} & & \text{HOCH} \\
| & & | & & | \\
\text{HCOH} & & \text{HCOH} & & \text{HCOH} \\
| & & | & & | \\
\text{HCOH} & & \text{HCOH} & & \text{HCOH} \\
| & & | & & | \\
\text{CH}_2\text{OH} & & \text{CH}_2\text{OH} & & \text{CH}_2\text{OH}
\end{array}
$$

D-Fructose   D-Glucitol   D-Mannitol
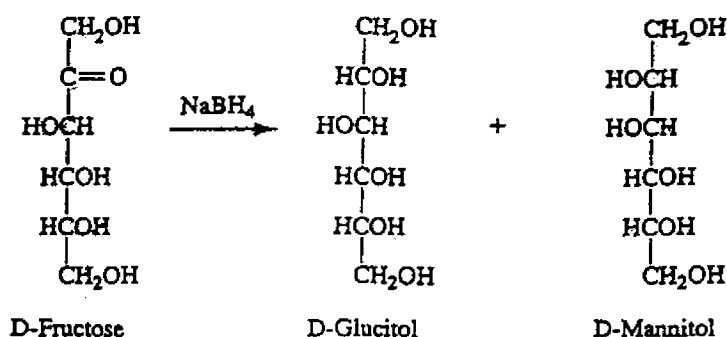
11-6 figure   Reduction of D-fructose to a mixture of alditols.

11.3.4.2.2 Hydrolyzates of Polysaccharides Containing Uronic Acids: Outline of Procedure (31)

A method different from that used for neutral sugars (section 11.3.4.2.1) is required when uronic acids are present.

1. **Reduction.** As with hydrolyzates containing only neutral sugars, the hydrolyzate is evaporated to dryness. The residue is dissolved in sodium carbonate solution and treated with an excess of sodium borohydride. Excess borohydride is decomposed by addition of glacial acetic acid, and borate is removed by addition and evaporation of methanol (section 11.3.4.2.1). This procedure reduces uronic acids to aldonic acids and aldoses to alditols (Fig. 11-5).

2. **Preparation and Chromatography of Trimethylsilyl (TMS) Derivatives.** The aldonic acids are converted into per-TMS ethers rather than peracetate esters (Fig. 11-5). Several procedures and packaged reagents have been developed for this etherification. The reaction mixture is injected directly into the chromatograph. Temperature programming is required. Components are identified by their retention times relative to that of docosane.

11.3.4.3 Enzymatic Methods

11.3.4.3.1 Overview

The method of choice for the determination of starch employs a combination of enzymes in sequential enzyme-catalyzed reactions and is specific for starch, providing purified enzyme preparations are used (see section 11.4.1.1).

Other enzymatic methods for the determination of carbohydrates have been developed (Table 11-3) (see also Chapter 22). They are often, but not always, specific for the substance being measured. Kits for several enzymatic methods have been developed and marketed by biochemical supply companies. The kits contain enzymes, other required reagents, buffer salts, and detailed instructions that should be followed because enzyme concentration, substrate concentration, concentration of other required reagents, pH, and temper-

ature all affect reaction rates and results. A good method will point out any interferences and other limitations.

Methods involving enzyme- or coupled enzyme-catalyzed reactions generally have low detection limits. They also are usually quite specific, although not always 100% specific. However, it is not often that determination of a single component is desired, the notable exception being the determination of starch (section 11.4.1.1). Other exceptions are the identification and quantitative determination of β-glucan and inulin (see section 11.4.2.2). What is most often sought is determination of total carbohydrate, or a class of carbohydrate, or most often, all carbohydrates individually. Thus, chromatographic methods (sections 11.3.4.1 and 11.3.4.2) that give values for each of the sugars present are preferred.

11.3.4.3.2 Sample Preparation

It sometimes is recommended that the Carrez treatment (4), which breaks emulsions, precipitates proteins, and absorbs some colors, be applied to food products prior to determination of carbohydrates by enzymatic methods. The Carrez treatment involves addition of a solution of potassium hexacyanoferrate ($K_4[Fe(CN)_6]$, potassium ferrocyanide), followed by addition of a solution of zinc sulfate ($ZnSO_4$), followed by addition of a solution of sodium hydroxide. The suspension is filtered, and the clear filtrate is used directly in enzyme-catalyzed assays.

11.4 POLYSACCHARIDES

11.4.1 Starch

Starch is second only to water as the most abundant component of food. A variety of commercial starches are available worldwide as food additives. These include corn (maize), waxy maize, high-amylose corn,

11-3

table | Selected Enzymatic Methods of Carbohydrate Analysis

Carbohydrate	Reference	Kit Form[1]
Monosaccharides		
Pentoses		
L-Arabinose	32,33	
D-Xylose	32,33	
Hexoses		
D-Fructose	32,33	x
D-Galactose	32,33	x
D-Galacturonic acid	32	
D-Glucose		
Using glucose oxidase	33, section 11.4.1.1	x
Using glucose dehydrogenase	32,33	
Using glucokinase (hexokinase)	32,33	x
D-Mannose	32,33	
Monosaccharide Derivatives		
D-Gluconate/D-glucono-δ-lactone	32,33	x
D-Glucitol/sorbitol	32,33	x
D-Mannitol	32,33	
Xylitol	32,33	x
Oligosaccharides		
Lactose	32,33	x
Maltose	32,33	x
Sucrose	32,33	x
Raffinose, stachyose, verbascose	32,33	x
Polsaccharides		
Amylose, amylopectin (contents and ratio)		x
Cellulose	32,33	
Galactomannans (guar and locust bean gums)	32	
β-Glucan (mixed-linkage)	32	x
Glycogen	32,33	
Hemicellulose	32,33	
Inulin	32,33	x
Pectin/poly(D-galacturonic acid)	32,33	
Starch	section 11.4.1.1, 32,33	x

[1]Available in kit form from companies such as Boehringer Mannheim, Megazyme, and Sigma Chemical Co.

potato, wheat, rice, barley, tapioca (cassava), arrowroot, and sago starches. In addition, starch is the main component of flours such as wheat, rye, barley, oat, rice, corn, and pea flours. Starch is also found in all parts of plants (leaves, stems, roots, tubers, seeds).

11.4.1.1 Total Starch

11.4.1.1.1 Principle The only reliable method for determination of total starch is based on total conversion of the starch into D-glucose by purified enzymes specific for starch, and determination of the D-glucose released by an enzyme specific for it (Fig. 11-7) (see also Chapter 22).

11.4.1.1.2 Potential Problems The starch-hydrolyzing enzymes (amylases) must be purified to eliminate any other enzymatic activity that would release D-glu-

cose (cellulases, for example) and catalase, which would reduce the stability of the dye complex. The former contamination would give false high values and the latter, false low values. Even with purified enzymes, problems can be encountered with this method. It may not be quantitative for high-amylose starch or other starch at least partially resistant to enzyme-catalyzed hydrolysis. Resistant starch, by definition, is composed of starch and starch-degradation products that escape digestion in the small intestine (34). There are three starch sources that are resistant to digestion or so slowly digested that they pass through the small intestine: (a) starch that is physically inaccessible to amylases because it is trapped within a food matrix; (b) starch that resists enzyme-catalyzed hydrolysis because of the nature of the starch granule (raw potato and banana starches are examples); and (c) retrograded starch, i.e., starch polymers that have recrystallized

TOTAL STARCH

Starch

| Gelatinization

Starch solution

| α-Amylase

Linear and branched
fragments of amylose
and amylopectin

| Glucoamylose

D-Glucose
| 1. Glucose oxidase
| 2. Peroxidase and
| a leuco dye

Color

DEGREE OF GELATINIZATION

Starch solution/suspension

| Debranching enzyme

Linear segments of amylose
and amylopectin

| β-Amylase

Maltose (primarily)

| Reducing sugar
| determination

Compare value with that
obtained for completely
gelatinized starch

11-7 figure Flow sheets for determination of total starch (section 11.4.1.1) and determination of the degree of starch gelatinization (section 11.4.1.2).

after gelatinization of the granules (for example, cooled cooked potatoes may contain resistant starch) (section 11.4.1.3).

$$
\begin{array}{l}
HC=O \\
| \\
HCOH \\
| \\
3\ HOCH \\
| \\
HCOH \\
| \\
HCOH \\
|
\end{array}
\quad + \quad
\text{[Sodium 3,5-Dinitrosalicylate]}
\quad + \quad 3\ OH^- \longrightarrow
\qquad [4]
$$
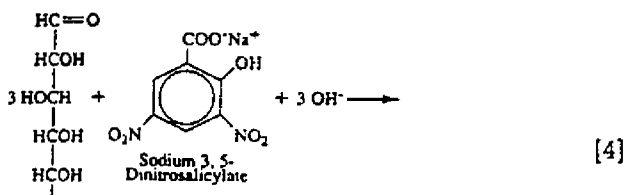
One method of starch analysis purports to overcome these problems (35). In it, the starch is dispersed in dimethyl sulfoxide (DMSO), and then is converted quantitatively to D-glucose by treatment with thermostable α-amylase to effect depolymerization and solubilization of the starch. Glucoamylase (amyloglucosidase) effects quantitative conversion of the fragments produced by the action of α-amylase into D-glucose, which is determined with a glucose oxidase/peroxidase (GOPOD) reagent (AOAC Method 969.39, American Association of Cereal Chemists Method 76-13). This reagent contains a colorless (leuco) dye that is oxidized to a colored compound by the hydrogen peroxide (produced by the glucose oxidase-catalyzed oxidation of glucose, Equation [4]) in a reaction catalyzed by peroxidase. This method determines total starch. It does not reveal the botanical source of the starch or whether it is native starch or modified food starch. The botanical source of the starch may be determined microscopically (section 11.6.1) if the material being analyzed has not been cooked. Some information about modification also may be determined with a microscope.

11.4.1.1.3 Outline of Procedure

1. Finely milled sample in a test tube is wet with 80% vol/vol ethanol. DMSO is added to the ethanol-wetted sample, and the contents of the tube are mixed vigorously. Then, the tube is heated in a boiling water bath.

2. Thermostable α-amylase solution is added with vortex mixing, and the tube is returned to the boiling water bath.

3. After 5 min, the tube is brought to 50°C. Sodium acetate buffer, pH 4.5, and glucoamylase (amyloglucosidase) solution is added, and the contents are mixed. The tube then is incubated at 50°C.

4. The tube contents are transferred quantitatively to a volumetric flask using distilled water to wash the tube and to adjust the contents to volume.

After thorough mixing of the flask, aliquots are removed, treated with GOPOD reagent, and incubated at 50°C. Absorbance of the test sample and a reagent blank is measured at the wavelength required by the GOPOD reagent being used.

Glucose and starch samples low in protein and lipid, such as potato starch, of known moisture content are used as standards. Addition of DMSO can be omitted, and the diluted thermostable α-amylase solution can be added directly to the ethanol-wetted sample if it is known from experience that no starch resistant to the α-amylase under the conditions used is present in the samples being analyzed.

11.4.1.2 Degree of Gelatinization of Starch

When starch granules are heated in water to a temperature specific for the starch being cooked, the granules swell, lose their crystallinity and birefringence, and become much more susceptible to enzyme-catalyzed hydrolysis. Heating starch in water produces phenomena that result from two processes: gelatinization and pasting, often referred to simply as gelatinization. They are very important in determining the texture and digestibility of foods containing starch.

Several methods have been developed that make use of the fact that certain enzymes act much more rapidly on cooked starch than they do on native starch. A particularly sensitive method employs a combination of pullulanase and β-amylase, neither of which is able to act on uncooked starch granules (36). With gelatinized or pasted starch, the enzyme pullulanase, which cleaves 1,6 linkages, debranches amylopectin and any branched amylose molecules, giving a mixture of linear segments of various sizes. (Isoamylase, another debranching enzyme, also may be used.) β-

Amylase then acts on the linear chains, converting them into maltose (Fig. 11-7). The degree of gelatinization is determined by measuring the amount of reducing sugar formed (section 11.3.3).

11.4.1.3 Degree of Retrogradation of Starch

Upon storage of a product containing cooked starch, two starch polymers, amylose and amylopectin, associate with themselves and with each other in polycrystalline arrays. This process of reordering is called retrogradation. (Retrogradation is a contributing factor to the staling of bread and other bakery products, for example.) Retrograded starch, like native starch, also is acted on only very slowly by the enzyme combination pullulanase plus β-amylase. Therefore, the method described in section 11.4.1.2 can be used to determine retrogradation. The decrease in reducing power (from maltose released by action of the enzyme combination) after storage is a measure of retrogradation.

11.4.2 Nonstarch Food Gums/Hydrocolloids

11.4.2.1 Overview

A starch or starches may be used as ingredients in a food product, either as isolated starch or as a component of a flour, or may occur naturally in a fruit or vegetable tissue. Other polysaccharides are almost always added as ingredients, although there are exceptions. These polysaccharides, along with the protein gelatin, comprise the group of ingredients known as food gums or hydrocolloids. Their use is widespread and extensive. They are used in everything from processed meat products to chocolate products, from ice cream to salad dressings, etc.

Analytical methods are required for these polysaccharides to enable both suppliers and food processors to determine the purity of a gum product, to ensure that label declarations of processors are correct, and to monitor that gums have not been added to standardized products in which they are not allowed. It also may be desirable to determine such things as the β-glucan content of oat or barley flour or breakfast cereal for a label claim or the arabinoxylan content of wheat flour to set processing parameters. Another processor may want to determine other polysaccharides not declared on the ingredient label such as those introduced by microorganisms during fermentation, for example, in making yogurt-based products.

Food gum analysis is problematic because polysaccharides present a variety of chemical structures, solubilities, and molecular weights. Plant polysaccharides do not have uniform, repeating-unit-type structures; rather their structures vary from molecule to molecule. In addition, the average structure can vary with the source and the conditions under which the plant is grown. Some polysaccharides are neutral; some are anionic. Some are linear; some are branched. Some of the branched polysaccharides are still effectively linear; some are bushlike. Some contain, in addition to sugar units, ether, ester, and/or cyclic acetal groups, either naturally or as a result of chemical modification. Some are soluble in cold water; some are soluble only in hot water; and some require aqueous solutions of acids, bases, or metal ion-chelating compounds to dissolve them. Some, like cellulose, are insoluble in anything but very special solvents. Polysaccharide preparations always are composed of a mixture of molecules with a range of molecular weights. All this structural diversity complicates qualitative analysis of food gums when their nature is unknown or when more than one is present. Structural heterogeneity complicates quantitative analysis.

Current methods depend on extraction of the gum(s), followed by fractionation of the extract. Separation invariably results in some loss of material. Most often, isolated gum is identified by identifying and quantitating its constituent sugars after acid-catalyzed hydrolysis. However, sugars are released from polysaccharides by hydrolysis at different rates and are destroyed by hot acids at different rates, so the exact monosaccharide composition of a polysaccharide may be very difficult to determine. The problems associated with the determination of gums in foods and various procedures that have been used have been reviewed (30,37,38).

Qualitative identification tests, specifications, and analytical methods for many food-approved gums/ hydrocolloids, including modified starches, have been established for the United States (39) and Europe (40). None of the qualitative methods are conclusive. AOAC International has established methods for analysis of some specific food products. Not all gums approved for food use are included; not all methods that determine total gums can be used if starch is present, and not all methods can be used to determine all gums. Hydrocolloid/gum suppliers and food processors often have their own specifications of purity and properties.

11.4.2.2 Gum/Hydrocolloid Content Determination

Several schemes, some published, some unpublished, have been developed for analysis of food products for food gums. Most are targeted to a specific group of food products, as it is difficult, perhaps impossible, to develop a universal scheme. A general scheme that is reported to work successfully (31) is presented here. Figure 11-8 presents the scheme for isolation and purification of water-soluble polysaccharides. Letters in the parentheses below refer to the same letters in Fig.

Sample

Freeze dry (a)

Dry sample

Fat extract (a)

Fat-free sample   aqueous ethanol extract (b) ——→   Soluble sugars, other
                                                     low-molecular-weight
Protein digestion (c)   compounds, and ash

Protein-free sample

Ethanol precipitation (d)

Polysaccharide precipitate

Starch digestion (e)

Starch-free sample

Centrifugation (e)

Insoluble fiber   Soluble fiber

Precipitation (f)

Polysaccharide precipitate

Dialysis (g)

Polysaccharide extract

Freeze dry (g)

Dry polysaccharide extract

Hydrolysis (h)

Derivatization

HPLC   GC
analysis   analysis

11-8

figure

Flow sheet for isolation and analysis of polysaccharides.

11-8. Several of the steps in the method utilize principles previously described.

(a) It is difficult to extract polysaccharides quantitatively when fats, oils, waxes, and proteins are present. Lipid-soluble substances are removed first. Before this can be effected, the sample must be dried. Freeze drying is recommended. If the dried material contains lumps, it must be ground to a fine consistency. A known weight of dry sample is placed in a Soxhlet apparatus, and the lipid-soluble substances are removed with 95:5 vol/vol chloroform–methanol. (n-Hexane also has been used, but a more polar solvent is recommended). Solvent is removed from the sample by air-drying in a hood, then by placing the sample in a vacuum dessicator.

(b) Although not in the published scheme, soluble sugars, other low-molecular-weight compounds, and ash can be removed at this point using hot 80% ethanol as described in section 11.3.1. (Hot 80% methanol also has been used.)

(c) Protein is removed by enzyme-catalyzed hydrolysis. The cited procedure (31) uses papain as the protease. One must always be aware of the fact that commercial enzyme preparations almost always have carbohydrase activities in addition to proteolytic activity. Thus, bacterial alkaline proteases are recommended by some because carbohydrases have acidic pH optima.

In this procedure (31), proteins are denatured for easier digestion by dispersion of the sample in sodium acetate buffer, pH 6.5, containing sodium chloride, and heating the mixture. Papain (activated by dispersing it in sodium acetate buffer, pH 6.5, containing cysteine and EDTA) is added to the sample, and the mixture is incubated.

(d) Any solubilized polysaccharides are precipitated by addition of sodium chloride solution to the cooled dispersion, followed by 4 volumes of absolute ethanol. The mixture is centrifuged.

(e) The pellet is suspended in acetate buffer, usually

pH 4.5. To this suspension is added a freshly prepared solution of glucoamylase/amyloglucosidase in the same buffer. This suspension is then incubated. Just as in the analysis of starch, highly purified enzyme must be used to minimize hydrolytic breakdown of other polysaccharides (see section 11.4.1.1). This step may be omitted if it is known that no starch is present. Centrifugation after removal of starch removes and isolates insoluble fiber (cellulose, some hemicelluloses, lignin).

The presence of starch can be tested for by adding a solution of iodine in potassium iodide solution and observing the color. A color change to brownish-red or blue indicates the presence of starch. A microscope may be used to a look for stained intact or swollen granules or granule fragments (section 11.6.1). However, unless a definite blue color appears, the test may be inconclusive. A better check is to analyze the ethanol-soluble fraction from step (f) for the presence of glucose (section 11.3.4). If no glucose is found, the starch digestion part of step (e) may be omitted in future analyses of the same product.

(f) Solubilized polysaccharides again are precipitated by addition of sodium chloride solution to the cooled dispersion, followed by 4 volumes of absolute ethanol. The mixture is centrifuged. The insoluble residue (pellet) is the insoluble dietary fiber (primarily cellulose and lignin) (section 11.5).

(g) The pellet is suspended in deionized water, transferred to dialysis tubing, and dialyzed against frequent changes of sodium azide solution. (Sodium azide is used to prevent microbial growth). Finally, the tube contents are dialyzed against deionized water to free it from sodium azide. The retentate is recovered from the dialysis tubing and freeze-dried.

(h) Polysaccharide identification relies on hydrolysis to constituent monosaccharides and identification of these sugars (section 11.3.4). For hydrolysis, polysaccharide material is added to a Teflon-lined, screw-capped vial. Trifluoroacetic acid solution is added, and the vial is tightly capped and heated. After cooling, the contents are evaporated to dryness in a hood with a stream of air or nitrogen. Then, sugars are determined by HPLC (section 11.3.4.1) or GC (section 11.3.4.2). If GC is used, inositol is added as an internal standard. Qualitative and quantitative analysis of the polysaccharides present can be determined from the sugar analysis. For example, guaran, the polysaccharide component of guar gum, yields D-mannose and D-galactose in a molar ratio of 1.00:0.56.

This acid-catalyzed hydrolysis procedure does not release uronic acids quantitatively. The presence of uronic acids can be indicated by either the modified carbazole assay (41,42) or the m-hydroxydiphenyl assay (4,43,44). Both methods are based on the same principle as the phenol–sulfuric acid assay (section 11.3.2.1), i.e., condensation of dehydration products

with a phenolic compound to produce colored compounds that can be measured quantitatively by means of spectrophotometry. With uronic acids, decarboxylation accompanies dehydration.

11.4.2.3 Pectin

11.4.2.3.1 Nature of Pectin Pectin is a very important food polysaccharide, yet no official methods for its determination have been established. What few methods have been published basically involve its precipitation with alcohol from jams, jellies, etc. in which it is the only polysaccharide present.

The definition of pectin is somewhat ambiguous. The structure of native pectin depends on the source, including the stage of development (degree of ripeness) of the particular fruit or vegetable. Generally, it can be described as a main chain of poly(methyl α-D-galactopyranosyluronate) interrupted by L-rhamnopyranosyl units (1,2). Many of the rhamnosyl units have arabinan, galactan, or arabinogalactan chains attached to them. Other sugars, such as D-apiose, also are present. In the manufacture of commercial pectin, much of the neutral sugars is removed. Commercial pectin is, therefore, primarily poly(α-D-galacturonic acid methyl ester) of various degrees of esterification, and sometimes amidation. Enzyme action during development/ripening or during processing can partially deesterify or depolymerize native pectin. Enzyme-catalyzed reactions are important determinants of the stability of fruit juices, tomato sauce and paste products, apple butter, etc. in which some of the texture is supplied by pectin and its interaction with calcium ions. It is probable that the fact that pectin is a moving target has precluded development of methods for its determination.

11.4.2.3.2 Pectin Content Determination The constant in pectins is D-galacturonic acid as the principal component (often at least 80%). However, glycosidic linkages of uronic acids are difficult to hydrolyze without decomposition, so methods involving acid-catalyzed hydrolysis and chromatography (section 11.4.2.1) are not applicable.

One method employed for pectin uses saponification in sodium hydroxide solution, followed by acidification, and addition of Ca^{2+} to precipitate the pectin. The calcium pectate is collected, washed, dried, and measured gravimetrically. Precipitation with a quaternary ammonium salt such as cetylpyridinium bromide, whose complex with pectin has a much lower critical electrolyte concentration than its complex with other acidic polysaccharides (45), has been used. However, pectin and other acidic polysaccharides are not likely to be found together. For a review of methods, see reference (46).

Because of the dominance of D-galacturonic acid in its structure, pectins are most often determined using the carbazole or m-hydroxydiphenyl methods (section 11.4.2.2). Isolation of crude pectin usually precedes analysis.

11.4.2.3.3 Degree of Esterification The degree of esterification (DE) is an important parameter in both natural products and added pectin. DE may be measured directly by titration before and after saponification. First, the isolated pectin (section 11.4.2.2) is washed with acidified alcohol to convert carboxylate groups into free carboxylic acid groups, then washed free of excess acid. Then, a dispersion of the pectinic acid in water is titrated with dilute base, such as standardized sodium hydroxide solution, to determine the percentage of nonesterified carboxyl ester groups. Excess base is added to saponify the methyl ester groups. Back-titration with standardized acid to determine excess base following saponification gives the DE. Also, methanol released by saponification can be measured directly by gas chromatography (47).

11.5 DIETARY FIBER

Dietary fiber (Chapter 12) is the sum of the nondigestible components of a foodstuff or food product. Most is plant cell wall material (cellulose, hemicelluloses, lignin). However, because only those polysaccharides in cooked starch are digestible (section 11.4.1.2), all other polysaccharides are components of dietary fiber. Some are components of insoluble fiber. Insoluble fiber components are cellulose, microcrystalline cellulose added as a food ingredient, hemicelluloses entrapped in a lignocellulosic matrix, and resistant starch (section 11.4.1.1.2). Others, including the majority of food gums/hydrocolloids (section 11.4.2), are classified as soluble fiber. Their determination often is important in terms of making food label claims and is described in section 11.4.2. Determination of the β-glucan content of products made with oat or barley flours is an example. In general, the pellet from centrifugation [step (e), Fig. 11-8] is insoluble fiber, and those components precipitated from the supernatant with alcohol [step (f), Fig. 11-8] constitute soluble fiber.

11.6 PHYSICAL METHODS

11.6.1 Microscopy

Microscopy can be a valuable tool in food analysis. Various kinds of microscopy (light, fluoresence, confocal, FTIR [Fourier transform infrared], scanning electron, transmission electron) have been used to study the organization of food products and the stability of

emulsions and foams, and to identify and quantitate extraneous matter (Chapter 23). Microscopy is particularly useful in examinations of starchy foods. Cell-wall fragments in flours can be seen with a light microscope, for example. More importantly, the plant source of a starch can be readily identified with a polarizing light microscope, since the morphologies and certain properties of starch granules are characteristic of the plant source. Granule size, shape, form, position of the hilum (botanical center of the granule), the degree of brightness under polarized light, and in some cases, iodine-staining characteristics are all inherent to the starch source (48).

In cooked starch products, the extent of retrogradation (49) and the effects of storage on microstructure have been evaluated by iodine staining and light microscopy (50-56). The degree that starch has been damaged mechanically during dry milling (57) and the extent of digestion by enzymes can be determined microscopically. Microscopy also can determine overcooking, undercooking, and correct cooking of products containing a starch product.

11.6.2 Spectrometry

In addition to the spectrophotometric methods already mentioned, near-infrared (NIR) transmittance spectrometry has been used to determine sugar content (57). NIR spectrometry is described in Chapter 27.

11.6.3 Specific Gravity (3)

Specific gravity is defined as the ratio of the density of a substance to the density of a reference substance (usually water), both at a specified temperature. The concentration of a carbohydrate solution can be determined by measuring the specific gravity of the solution and referring to appropriate specific gravity tables.

Measurement of specific gravity as a means of determining sugar concentration is accurate only for pure sucrose or other pure solutions (AOAC Method 932.14), but it can be, and is, used for obtaining approximate values for liquid products (see also Chapter 5, section 8.5.2). Two basic means of determining specific gravity are used. By far the most common is use of a hydrometer calibrated either in °Brix, which corresponds to sucrose concentrations by weight, or in Baumé Modulus (°Bé). A calibrated pycnometer also may be used.

11.6.4 Refractive Index (3)

When electromagnetic radiation passes from one medium to another, it changes direction, being bent or refracted. The ratio of the sine of the angle of incidence to the sine of the angle of refraction is termed the index

of refraction, or the refractive index (RI). The RI varies with the nature of the compound, temperature, wavelength of light, and concentration of the compound. By holding the first three variables constant, the concentration of the compound can be determined by measuring the RI. Thus, measurement of refractive index is another way to determine total solids in solution (see also Chapter 8, section 8.5.3). Like determination of specific gravity, use of RI to determine concentrations is accurate only for pure sucrose or other pure solutions, but also like the determination of specific gravity, is used for obtaining approximate sugar concentration values for liquid products. In this case, the solution must be clear. Refractometers that read directly in sucrose units are available.

11.6.5 Polarimetry (3)

Most compounds that contain a chiral carbon atom will rotate the plane of polarization of polarized light. A polarimeter measures the extent to which a compound in solution rotates the plane of polarized light. Carbohydrates have chiral carbon atoms, so they have optical activity. Carbohydrates rotate the plane of polarized light through an angle that depends on the nature of the compound, temperature, wavelength of light, and concentration of the compound. The concentration of the compound can be determined from a value known as the specific optical rotation if all other factors are held constant and if the solution contains no other optically active compounds.

Determination of specific optical rotation is used to measure sucrose concentration (AOAC Methods 896.02, 925.46, 930.37). Instruments are available that read in units of the International Sugar Scale. Determination of specific optical rotation before and after hydrolysis of sucrose into its constituent sugars, D-glucose and D-fructose, a process called inversion, can be used to determine sucrose in the presence of other sugars (AOAC Methods 925.47, 925.48, 926.13, 926.14).

11.7 SUMMARY

For determination of low-molecular-weight carbohydrates, older colorimetric methods for total carbohydrate and various reducing sugar methods largely have been replaced by chromatographic methods. The older methods suffer from the fact that they are not stoichiometric and, therefore, require standard curves. This makes them particularly problematic when a mixture of sugars is being determined. However, they are still used. Chromatographic methods (HPLC and GC) separate mixtures into the component sugars, identify each component by retention time, and provide a quantitative measurement of each component. Enzy-

matic methods are specific and sensitive, but seldom, except in the case of starch, is determination of only a single component desired.

Polysaccharides are important components of many food products. Yet there is no universal procedure for their analysis. Generally, isolation must precede measurement. Isolation introduces errors because no extraction or separation technique is quantitative. Identification and measurement are done by hydrolysis to constituent monosaccharides and their determination. An exception is starch, which can be digested to glucose using specific enzymes (amylases), followed by measurement of the glucose released.

11.8 STUDY QUESTIONS

1. Give three reasons why carbohydrate analysis is important.
2. Distinguish chemically between monosaccharides, oligosaccharides, and polysaccharides, and explain how solubility characteristics can be used in an extraction procedure to separate monosaccharides and oligosaccharides from polysaccharides.
3. Discuss why mono- and oligosaccharides are extracted with 80% ethanol rather than with water. What is the principle involved?
4. Define reducing sugar. Classify each of the following as a reducing or nonreducing carbohydrate: D-glucose, D-fructose (Conditions must be described. Why?), sucrose, maltose, raffinose, maltotriose, cellulose, amylopectin.
5. Briefly explain one method that could be used for each of the following:
   a. to prevent hydrolysis of sucrose when sugars are extracted from fruits via a hot alcohol extraction
   b. to remove proteins from solution for an enzymatic analysis
   c. to measure total carbohydrate
   d. to measure total reducing sugars
   e. to measure the sucrose concentration of a pure sucrose solution by a physical method
   f. to measure glucose enzymatically
   g. to measure simultaneously the concentrations of individual free sugars
6. What are the principles behind total carbohydrate determination using the phenol–sulfuric acid method? Give an example of another assay procedure based on the same principle.
7. What is the principle behind determination of total reducing sugars using the Somogyi–Nelson and similar methods?
8. The Munson–Walker, Lane–Eynon, and Somogyi–Nelson methods can be used to measure reducing sugars. Explain the similarities and difference among these methods with regard to the principles involved and the procedures used.
9. Describe the principle behind anion-exchange chromatography of carbohydrates.
10. Describe the general procedure for preparation of sugars

for gas chromatography. What is required for this method to be successful?

11. What difference is there between the preparation of an extract of reducing sugars for gas chromatography and the preparation of polysaccharide hydrolyzates containing uronic acids for gas chromatography? What two differences are there in the final derivatives?

12. Why has HPLC largely replaced GC for analysis of carbohydrates?

13. Compare and contrast RI and PAD detectors.

14. What is the advantage of an enzymatic method? What is the limitation (potential problem)?

15. Describe the principles behind the enzymatic determination of starch. What are the advantages of this method? What are potential problems?

16. Describe the principle behind each step in Fig. 11-8. What is the reason for each step?

17. Describe the principles behind separation and analysis of cellulose, water-soluble gums, and starch.

18. Describe two methods for determination of pectin.

19. Describe the principles behind and the limitations of determining sugar (sucrose) concentrations by (a) specific gravity determination, (b) refractive index measurement, and (c) polarimetry.

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Fiber Analysis

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12.1 INTRODUCTION

12.1.1 Importance of Dietary Fiber

In the early 1970s; Burkitt and Trowell (1) postulated that the prevalence of heart disease and certain cancers in Western societies was related to inadequate consumption of dietary fiber. Their observations stimulated much interest, and a great deal of research has been done to test the fiber hypothesis. While the research has not always produced consistent results and the great expectations inspired by Burkitt and Trowell have not materialized, it is clear that adequate consumption of dietary fiber is important for optimum health.

Liberal consumption of dietary fiber from a variety of foods will help protect against colon cancer and also help normalize blood lipids, thereby reducing the risk of cardiovascular disease. Certain types of fiber can slow glucose absorption and reduce insulin secretion, which is of great importance for diabetics and probably for nondiabetics as well. Fiber helps prevent constipation and diverticular disease. With this wide range of beneficial effects attributable to dietary fiber, it is easy to lose perspective and consider dietary fiber a magic potion that will correct or prevent all diseases. A more correct view is that dietary fiber is an essential component of a well-balanced diet, and adequate intake of dietary fiber throughout one's lifetime will help minimize some of the most common health problems in the United States. References (2–5) provide an extensive compilation of articles related to the physiological action of dietary fiber and resistant starch.

The Daily Reference Value (DRV) for dietary fiber has been set at 25 g per 2000 kcal to promote optimal health. However, dietary fiber includes a variety of fibrous materials that in turn produce a variety of physiological actions (2–5). For example, the pentose fraction of dietary fiber seems to be the most beneficial in preventing colon cancer and reducing cardiovascular disease. Pectin and the hydrocolloids are most beneficial in slowing glucose absorption and lowering insulin secretion. Pectins and hydrocolloids are of little value in preventing diverticulosis and constipation; however, a mixture of hemicellulose and cellulose will help prevent these gastrointestinal dysfunctions. Recognition of the importance of dietary fiber and of the fact that certain physiologic effects can be related to specific fiber components has led to the emergence of a number of methodologies for determining dietary fiber. Two commonly used methods for analyzing dietary fiber are described in this chapter.

12.1.2 Definition of Fiber

Dietary fiber is most frequently defined as polysaccharides and lignin that are not digested by human enzymes (6). Most starch is readily digestible by human glucosidases. However, some starch escapes digestion in the small intestine and the starch that is not digested is called resistant starch. Since resistant starch is a plant polysaccharide, it technically fits the definition of dietary fiber. However, another common definition of dietary fiber is lignin plus plant nonstarch polysaccharides. By this definition resistant starch is not a component of dietary fiber.

12.1.3 Major Components of Dietary Fiber

The major components of dietary fiber are cellulose, hemicelluloses, pectins, hydrocolloids, and lignin. From a botanical view, fiber is categorized as cell wall polysaccharides, non-cell-wall polysaccharides, and lignin.

12.1.3.1 Cell Wall Polysaccharides

12.1.3.1.1 Cellulose Cellulose is a long, practically linear polymer of α-1,4-linked glucose units. Some polymers may contain 10,000 glucose units. Hydrogen bonding between parallel polymers forms strong microfibrils. Cellulose microfibrils provide the strength and rigidity required in plant primary and secondary cell walls.

12.1.3.1.2 Hemicelluloses Hemicelluloses are a heterogeneous group of substances containing a number of sugars in their backbone and side chains. Xylose, mannose, and galactose frequently form the backbone structure, while arabinose, galactose, and uronic acids are present in the side chains. Hemicelluloses by definition are soluble in dilute alkali but not in water. Molecular size and degree of branching also are highly variable. A typical hemicellulose molecule contains 50–200 sugar units. Hemicelluloses are matrix polysaccharides that tie together cellulose microfibrils and form covalent bonds with lignin.

12.1.3.1.3 Pectins Pectins are rich in uronic acids. They are soluble in hot water and form gels. The backbone structure consists of unbranched chains of 1,4-linked galacturonic acid. Side chains may contain rhamnose, arabinose, xylose, and fucose. Solubility is reduced by methylation of the free carboxyl group and by formation of calcium and magnesium complexes. Pectins, like hemicelluloses, are matrix polysaccharides in cell walls.

12.1.3.2 Non-Cell-Wall Polysaccharides

Non-cell-wall polysaccharides include hydrocolloids such as mucilages, gums, and algal polysaccharides. Hydrocolloids are hydrophilic polysaccharides that

form viscous solutions or dispersions in cold or hot water. Typical mucilages are guar and locust bean gums. Oats and barley also contain mucilages. Plant exudate gums include Arabic, Ghatti, Karaya, and Tragacanth gums, while algal polysaccharides include agar, alginates, and carrageenan. The non-cell-wall polysaccharides contain a variety of neutral sugars and uronic acids.

12.1.3.3 Lignin

Lignin is a noncarbohydrate, three-dimensional polymer consisting of approximately 40 phenol units with strong intramolecular bonding. Lignin often is covalently linked to hemicellulose.

12.2 GENERAL CONSIDERATIONS

Fiber components or subfractions are methodology dependent and not distinct entities. These fractionations are somewhat arbitrary and frequently have little relationship to either plant or mammalian physiology. Although considerable progress has been made in relating fiber composition to physiological action during the past 20 years, much remains to be learned. Pectins and hydrocolloids have long been used as additives in food processing. However, improving the nutritional value of foods by adding fiber or modifying resistant starch content remains a challenge for the food scientist.

12.3 METHODS

12.3.1 Overview

Dietary fiber is estimated by two basic approaches—gravimetric or chemical. In the first approach, digestible carbohydrate, lipids, and proteins are selectively solubilized by chemicals and/or enzymes. Undigestible materials then are collected by filtration, and the fiber residue is quantitated gravimetrically. In the second approach, digestible carbohydrates are removed by enzymatic digestion, fiber components are hydrolyzed by acid, and monosaccharides are measured. The sum of monosaccharides in the acid hydrolysate represents fiber.

Southgate (7,8) was the first to systematically quantitate dietary fiber in a wide range of foods. The carbohydrate chemistry used by Southgate has been improved and modernized, but his approach forms the foundation for many of the currently used gravimetric and chemical methods for fiber determination.

The food component that is most problematic in fiber analysis is starch. In both gravimetric and chemi-

cal approaches, it is essential that all starch be removed for accurate estimates of fiber. With the gravimetric approach, incomplete removal of starch increases the residue weight and inflates the estimate of fiber. In the chemical approach, glucose in the acid hydrolysate is considered fiber. Therefore, glucose that is not removed in the early analytical steps causes an overestimation of dietary fiber. The starch hydrolases utilized in fiber methods include α-amylase, amyloglucosidase, and pullulanase. α-Amylase catalyzes the hydrolysis of internal α-1,4-linked D-glucose units, while pullulanase hydrolyzes internal α-1,6-linked glucose units. Amyloglucosidase hydrolyzes α-1,4- and α-1,6-glucosidic bonds from nonreducing ends of starch. Takadiastase™ is a heat-stable fungal α-amylase, and Termamyl™ is a heat-stable bacterial α-amylase.

All fiber methods include a heating step at 80–130°C for 10 min to 3 hr to swell and disintegrate (gelatinize) starch granules. Even with gelatinization, resistant starch (retrograded starch, starch associated with Maillard reactions, and highly crystalline starch) is not hydrolyzed by glucosidases. Thus, resistant starch is measured as fiber in the gravimetric procedures unless the analyst specifically corrects for resistant starch.

In the gravimetric approach, it is essential that either all digestible materials be removed from the sample so that only undigestible polysaccharides remain or that the undigestible residue be corrected for remaining digestible contaminants. Lipids are removed easily from the sample with organic solvents and generally do not pose analytical problems for the fiber analyst. Protein and minerals that are not removed from the sample during solubilization steps should be corrected by Kieldahl nitrogen analysis (see Chapter 15) and by ashing (see Chapter 9) portions of the fiber residue.

The descriptions of the various specific procedures in this chapter are meant to be overviews of the methods. The reader is referred to the referenced original articles for specifics regarding chemicals, reagents, apparatus, and step-by-step instructions.

12.3.2 Sample Preparation

Estimates of fiber are most consistent when the samples are low in fat (less than 5–10% fat), dry, and finely ground. If the sample contains more than 10% fat, extract fat by mixing the sample with 25 parts (vol/wt) petroleum ether or hexane. Centrifuge and decant the organic solvent. Repeat the lipid extraction two more times. Dry the sample overnight in a vacuum oven at 70°C and grind to pass through a 0.3–0.5 mm mesh screen. Record loss of weight due to fat and moisture removal and make appropriate correction to the final percentage dietary fiber value found in the analysis.

Nonsolid samples less than 10% fiber are best analyzed after lyophilization and treated as described pre-

viously. Nonsolid samples greater than or equal to 10% fiber can be analyzed without drying if the sample is homogeneous and low in fat and if particle size is sufficiently small to allow efficient removal of digestible carbohydrate and protein.

12.3.3 Gravimetric Methods

12.3.3.1 Crude Fiber

The crude fiber method was developed in the 1850s to estimate undigestible carbohydrate in animal feeds. Since an easy alternative was not available, fiber in human foods was measured as crude fiber until the early 1970s (except for Southgate in England). Crude fiber is determined by sequential extraction of the sample with 1.25% H_2SO_4 and 1.25% NaOH. The insoluble residue is collected by filtration and the residue is dried, weighed, and ashed to correct for mineral contamination of the fiber residue. Crude fiber measures variable amounts of the cellulose and lignin in the sample, but hemicelluloses, pectins, and the hydrocolloids are solubilized and not detected. Therefore, crude fiber determinations should be discontinued.

12.3.3.2 Detergent Methods

The acid detergent fiber and neutral detergent fiber methods were developed to more accurately estimate lignin, cellulose, and hemicellulose in animal feeds. Acid detergent fiber measures lignin and cellulose in the sample. Neutral detergent fiber is equal to acid detergent fiber plus hemicelluloses. Neither method measures pectins and hydrocolloids. Since pectins and hydrocolloids tend to be minor constituents in most feedstuffs, the detergent methods were quite adequate and well accepted for animal industries. The neutral detergent fiber method was the forerunner of the current American Association of Cereal Chemists (AACC) method (9) for determining insoluble fiber (AACC Method 32–20). Since pectins and hydrocolloids are important to human health, it is difficult to justify using these methods any longer to analyze foods.

12.3.3.3 Total, Insoluble, and Soluble Fiber

With the recognition that insoluble fiber and soluble fiber produce quite different physiological responses and that both types of fiber are important to human health, a number of methods with only minor differences were simultaneously proposed. A common procedure was developed from the earlier ones into what is now the widely accepted method of AOAC International (AOAC Method 991.43) (10). This method represents a slow evolution of methodologies that combined crude fiber, detergent fiber, and Southgate methodologies.

12.3.3.3.1 Principle
Dry, fat-extracted ground food samples are enzymatically digested with α-amylase, amyloglucosidase, and protease to remove starch and protein. Insoluble fiber is collected by filtration. Soluble fiber is precipitated by bringing the filtrate to 78% ethanol and collected by filtration. The filtered fiber residues are washed with ethanol and acetone, oven dried, and weighed. The fiber residues are analyzed for protein and ash content [fiber = residue wt – (wt of protein + wt of ash)].

12.3.3.3.2 Procedure
A flow diagram outlining the general procedure for the AOAC method of determining total, insoluble, and soluble dietary fiber is shown in Fig. 12-1. Samples are mixed with buffer, a heat-stable α-amylase is added, and the pH is adjusted. Starch is gelatinized and digested by heating the digestion mixture in a boiling water bath. After cooling, a protease enzyme is added to digest protein. After protein digestion, the pH is adjusted and starch digestion is completed with amyloglucosidase.

The next few steps differ depending on whether total, insoluble, or soluble fiber is being determined. If total fiber is to be determined without partitioning fiber into soluble and insoluble fractions, proceed as described in the note at the bottom of Fig. 12-1.

To determine insoluble and soluble fiber fractions, the digestion mixture following amyloglucosidase treatment is filtered through fritted crucibles containing Celite. The insoluble fiber retained by the filter is washed with water. The soluble fiber is in the filtrate. Four volumes (vol/vol) of 95% ethanol are added to the filtrate plus water washes to precipitate the soluble fiber. The precipitate is allowed to form and the mixture is vacuum filtered through fritted crucibles containing Celite. The soluble fiber residue is washed successively three times with 78% ethanol.

The fiber residue (total, insoluble, or soluble fiber) in the crucibles then is washed with 95% ethanol and acetone. The crucibles are oven dried, cooled, and weighed. Since some protein and minerals are complexed with plant cell wall constituents, fiber values must be corrected for these contaminants. If resistant starch is to be determined, triplicate samples should be analyzed. If resistant starch is not determined, duplicate samples are analyzed. One sample is used to determine nitrogen content by the Kjeldahl procedure, and another sample is incinerated to determine ash content. Resistant starch in fiber residue can be determined by suspending the residue in 2 M KOH (11). The base solubilizes resistant starch that then is digested by amyloglucosidase after the pH is adjusted to 4.0–4.7. Liberated glucose then is determined enzymatically or colorimetrically.

Duplicate reagent blanks must be run through the entire procedure for each type of fiber determination.

Food sample (1 g)
↓
Add 40 ml of buffer (pH 8.2)
Add heat-stable α-amylase
↓
Incubate 15 min at 95–100°C
↓
Cool to 60°C
↓
Add protease
↓
Incubate 30 min at 60°C
↓
Adjust pH to 4.0–4.7
Add amyloglucosidase
↓
Incubate 30 min at 60°C
↓
Filter the digest
↓
Wash filtered residue with 10 ml of water (2 times)
↓
Filtrate + water washes are used for soluble fiber determination (see note below)
↓

Insoluble Fiber
Wash residue with 10 ml of 95% ethanol (2 times)
↓
Wash residue with 10 ml of acetone (2 times)
↓
Oven dry
↓
(1a) Weigh crucible
↓
(1b) Ash one of the duplicates and reweigh
(525°C for at least 5 hr)
(1c) Determine residual protein on the other duplicate (Kjeldahl N x 6.25)
(1d) Calculate insoluble fiber content

Soluble Fiber[1]
Bring filtrate and water washes to 80 g with water
↓
Add 320 ml of 95% ethanol preheated to 60°C
↓
Precipitate formation (1 hr at room temp)
↓
Filter the digest
↓
Wash filtered residue with 20 ml of 78% ethanol (3 times)
↓
Wash residue with 10 ml of 95% ethanol (2 times)
↓
Wash residue with 10 ml of acetone (2 times)
↓
Oven dry
↓
(2a) Weigh crucible
↓
(2b) Ash one of the duplicates and reweigh
(2c) Determine residual protein on the other duplicate (Kjeldahl N x 6.25)
(2d) Calculate soluble fiber content
Total Dietary Fiber = Insoluble + Soluble fiber

[1]Total dietary fiber can be determined directly by weighing the digest following amyloglucosidase digestion and either (1) adding 4 volumes (vol/vol) of 95% ethanol preheated to 60°C or (2) adjusting the volume to 80 g with water and then adding 320 ml of 95% ethanol preheated to 60°C. After (1) or (2), follow the procedure for determining soluble fiber starting at the precipitate formation step. Calculation of (2d) would produce total dietary fiber.

12-1 figure   Method to determine total dietary fiber content of foods. AOAC method 991.43. [Adapted from (10).]

12-1 table — Dietary Fiber Sample and Blank Data Sheet

	SAMPLE		BLANK	
	Insoluble Fiber	Soluble Fiber	Insoluble Fiber	Soluble Fiber
Sample wt (mg)	m_1	m_2		
Crucible + Celite wt (mg)								
Crucible + Celite + residue wt (mg)								
Residue wt (mg)	R_1	R_2	P_1	R_2	R_1	R_2	R_1	R_2
Protein (mg) P								
Crucible + Celite + ash wt (mg)								
Ash wt (mg) A								
Blank wt (mg) B								
Fiber (%)								

Adapted with permission from (12). *The Journal of AOAC International*, 1988, 71(5):1019. Copyright 1988 by AOAC International.

$$\text{Blank (mg)} = \frac{R_1 + R_2}{2} - P - A$$

$$\text{Fiber (\%)} = \frac{\frac{R_1 + R_2}{2} - P - A - B}{\frac{m_1 + m_2}{2}} \times 100$$

Table 12-1 shows a sample and blank sheet used to calculate fiber percentages. Using the equations shown, percent dietary fiber is expressed on a dry weight basis if the sample weights are for a dried sample.

12.3.3.3.3 Applications The AOAC method for determining fiber has been tested extensively and found suitable for routine fiber analyses for research, legislation, and labeling purposes. Table 12-2 shows the fiber content of select foods analyzed by the AOAC method.

12-2 table — Total, Soluble, and Insoluble Dietary Fiber In Foods as Determined by the AOAC Method 991.43[1]

Food	Soluble	Insoluble	Total
Barley, dehulled, rolled	5.02	7.05	12.25
High-fiber cereal	2.78	30.52	33.73
Oat bran	7.71	9.73	16.92
Soy bran	6.90	60.53	67.14
Apricots	0.53	0.59	1.12
Prunes	5.07	4.17	9.29
Raisins	0.73	2.37	3.13
Carrots	1.10	2.81	3.93
Green beans	1.02	2.01	2.89
Parsley	0.64	2.37	2.66

Adapted from (10). *The Official Methods of Analysis*, 16th Ed. Copyright 1997 by AOAC International.
[1]Grams of fiber per 100 g of food on an as is, fresh weight basis.

Fiber residues can be utilized to determine resistant starch. This method can be used to determine fiber content of all foods.

12.3.4 Chemical Methods

12.3.4.1 Overview

In chemical methods for fiber determination, fiber is equal to the sum of all nonstarch monosaccharides plus lignin. Monosaccharides are measured either indirectly by colorimetric methods or by chromatographic [gas chromatography (GC) (Chapter 33) or high performance liquid chromatography (HPLC) (Chapter 32)] methods.

Carbohydrates in the presence of strong acids combine with a number of substances to produce chromogens that can be measured spectrophotometrically. Under specific, standardized conditions, hexoses can be measured with anthrone, pentoses with orcinol, and uronic acids with carbazole. There is mutual interference among groups of sugars that can and should be corrected mathematically (11). The sum of hexoses, pentoses, and uronic acids is taken as total polysaccharide content.

Uronic acid is technically difficult to measure by chromatography. Therefore, most procedures estimating fiber from monosaccharide analyses measure uronic acids colorimetrically by the carbazole method (13). The uronic acid values then are corrected for the presence of hexoses and pentoses as noted previously.

12.3.4.2 Englyst–Cummings Procedure

This procedure (14) is a modernized version of the Southgate procedure (7,8) and is a reasonable alternative to the AOAC method.

12.3.4.2.1 Principle Starch is gelatinized and enzymatically digested. The remaining nonstarch polysaccharides are hydrolyzed by sulfuric acid to liberate free monosaccharides. Neutral sugars are determined by GC and uronic acids are determined colorimetrically. An alternate, rapid procedure measures all monosaccharides by a colorimetric method (15). Values for total, soluble, and insoluble fiber can be determined by both approaches. With the GC procedure, fiber can be divided into cellulose and noncellulosic polysaccharides with values for constituent sugars.

12.3.4.2.2 Procedure A flow diagram of the Englyst–Cummings procedure is shown in Fig. 12-2. Samples containing ≤200 mg of dry matter are mixed with dimethyl sulfoxide and heated in a boiling water bath to gelatinize and disperse starch. Starch and protein are digested with pancreatin and pullanase. Starch digestion is completed by incubating the samples with amyloglucosidase. Fiber is precipitated by adding 100% ethanol and placing samples in a refrigerator. The fiber residue is collected by centrifugation and the supernatant is decanted as much as possible without disturbing the residue. The fiber is washed by resuspending the residue in 85% ethanol, and the residue is collected by centrifugation. Additional washes are done with 85% ethanol, 100% ethanol, and acetone. Then the fiber residue is dried. All supernatants from centrifugation steps are discarded.

Cellulose in the residue is hydrolyzed by mixing the dry residue with 12 M H_2SO_4 and heating. Noncellulosic polysaccharides are hydrolyzed by rapidly adding water, mixing, and heating in a boiling water bath. The acid hydrolysate is used for sugar analysis. A portion of the hydrolysate is used to derivatize neutral sugars for GC analysis, and a second aliquot is used for uronic acid determination by a colorimetric procedure. The rapid version of the Englyst–Cummings method [15] estimates monosaccharide content of the acid hydrolysate by a single colorimetric method. Fiber weight = monosaccharide weight in the rapid method or = neutral sugar + uronic acid weight in the GC method. No corrections are made for sugar losses during hydrolysis or for the addition of one molecule of water per glycoside bond since the corrections tend to offset each other.

The procedures just described yield total dietary fiber. To determine insoluble dietary fiber, the 40 ml of absolute ethanol is replaced with pH 7 buffer, and the water-soluble fiber is extracted by heating the digested

Food sample
(≤200 mg of dry matter)
↓
Add 2 ml of DMSO
Gelatinize at 100 °C for 30 min
↓
Add buffer containing
pancreatin and pullanase
↓
Digest for 10 min at 100°C
↓
Add amyloglucosidase
↓
Digest for 30 min at 50°C,
10 min at 100°C
↓
Add 40 ml of 100% ethanol¹
↓
Precipitate formation at 0°C for 30 min
↓
Centrifuge at 1000 × g for 10 min
↓
Wash precipitate by resuspending in 50 ml of 85% ethanol
Centrifuge at 1000 × g for 10 min
↓
Wash with 100% ethanol (2 times)
↓
Wash with acetone
↓
Dry residue
(place tubes in 65°C water
bath for several min)
↓
Add 5 ml of 12 M H_2SO_4
Hydrolyze cellulose for
1 hr at 35°C
↓
Make H_2SO_4 to 2 M by adding 25 ml of water
Hydrolyze 1 hr at 100°C
↓
Hydrolysate

Measure neutral | Measure uronic acids
sugars by GC | colorimetrically

Total Dietary Fiber = Neutral Sugars + Uronic Acids

¹To measure insoluble fiber, use 40 ml of buffer instead of 40 ml of ethanol, and extract soluble fiber at 100°C for 30 min. Continuation of the procedure yields insoluble fiber. Soluble fiber = total fiber – insoluble fiber

12-2 figure Englyst–Cummings procedure for determination of soluble, insoluble, and total dietary fiber. [Adapted from (14).]

mixture for 30 min in a boiling water bath. The insoluble fiber is collected by centrifugation, washed, dried, and hydrolyzed as described previously. Soluble fiber = total fiber – insoluble fiber. Cummings, Englyst, and Wood [16] provide details for measuring resistant starch.

The cellulose content of the total fiber can be obtained by omitting the hydrolysis step with 12 M H_2SO_4 and proceeding directly to hydrolysis of noncel-

lulose polysaccharides with 2 M H_2SO_4. Monosaccharide weight after hydrolysis with 2 MH_2SO_4 = noncellulosic polysaccharides. Cellulose content = total fiber – noncellulosic polysaccharides.

12.3.4.2.3 Applications The rapid colorimetric procedure is essentially a single-tube assay and does not require special analytical skills or equipment other than a colorimeter. The GC-based procedure can be used to provide more detail regarding chemical composition of the fiber in addition to quantitating fiber content. The Englyst–Cummings procedure does not measure and therefore does not include lignin as a component of total dietary fiber. Since most foods do not contain significant amounts of lignin, this is a suitable method for determining fiber content of most foods. For foods containing a significant quantity of lignin, the AOAC procedure or procedures described by Marlett [17,18] or Theander and Westerlund [19] should be used.

In the Englyst–Cummings procedure, resistant starch is not included as a component of fiber. The detailed Englyst–Cummings procedure [16] includes a step to measure resistant starch, but this step is not included in Fig. 12-2.

12.4 COMPARISON OF METHODS

The AOAC and the Englyst–Cummings methods are the most widely used procedures for determining dietary fiber. These and several other very similar methods all give quite comparable estimates of fiber content for a wide variety of foods. In general, the Englyst–Cummings method gives the lowest fiber values because lignin and resistant starch are not included as part of fiber in this method. Obviously, foods with a significant amount of resistant starch, such as corn flakes, and foods with significant lignin, such as cereal brans, will show the greatest deviation. The current AOAC method suggests that foods rich in simple sugars (glucose, fructose, and sucrose) be extracted with 85% ethanol. If sugars are not extracted from foods such as dry fruits and composite meals prior to fiber analysis, fiber content is overestimated. This does not appear to be a problem with the Englyst–Cummings procedure, possibly because of the small sample size relative to the large amount of ethanol used to precipitate soluble fiber. With the small sample size ($\leq$200 mg dry matter) in the Englyst–Cummings procedure, it is imperative that the food be completely homogeneous so that accurate subsamples can be taken for fiber analysis.

Both the AOAC and the Englyst–Cummings procedures incorporate a proteolytic enzyme to digest protein. Proteolysis allows some of the fiber to be solubilized, which in effect moves some of the insoluble fiber fraction into the soluble fiber fraction. In addition, proteolysis has the general effect of reducing the amount of material measured as lignin.

The AOAC procedure includes resistant starch as a dietary fiber component. Baked, flaked, and extruded products will have a significantly higher fiber value if determined by the AOAC procedure rather than by the Englyst–Cummings method. Correcting fiber values determined by the AOAC procedure for resistant starch produces fiber values more similar to fiber values determined by the Englyst–Cummings procedure.

The rapid Englyst–Cummings procedure requires the least amount of time, technical skill, and specialized equipment compared to the other commonly used methods. Overall, the Englyst–Cummings precedure is slightly more reproducible than the AOAC procedure.

Which fiber analysis method to choose is in part determined by (1) how much technical skill is available; (2) what the time constraints are; (3) availability of GCs and HPLCs; and (4) the importance of knowledge of constituent sugar composition, cellulose, noncellulose, pectin, or lignin content. If only total, soluble, and insoluble fiber analyses are needed, the AOAC and the rapid Englyst–Cummings methods are preferable. If the major components of fiber or constituent sugar composition are required, then the Englyst–Cummings GC procedure or the approaches used by Marlett [17,18] or Theander [19] would be the method of choice.

12.5 SUMMARY

Dietary fiber can be defined as polysaccharides and lignin that are indigestible by mammalian enzymes. An alternate definition of dietary fiber is lignin plus plant nonstarch polysaccharides. The major components of dietary fiber are cellulose, hemicellulose, pectin, lignin, and hydrocolloids (gums, mucilages, and algal polysaacharides). The crude fiber measurement drastically underestimates dietary fiber in foods since it measures only cellulose and lignin. All current methods use a combination of heat-stable α-amylase and amyloglucosidase to digest and remove starch from the sample. Gravimetric procedures then digest and remove protein with a protease. The remaining indigestible material (fiber) is collected by filtration and weighed. The fiber residue is corrected for residual protein and ash contamination.

Chemical procedures collect macromolecules in the amylase–amyloglucosidase digest by filtration with or without ethanol precipitation. The polysaccharides in the precipitate are hydrolyzed with sulfuric acid and quantitated colorimetrically or chromatographically. The combined weight of sugars in the acid hydrolysate is equal to fiber weight.

Filtration and precipitation of polysaccharides with ethanol at specific steps in both the gravimetric and chemical methodologies allow differentiation of soluble, insoluble, and total fiber.

Current gravimetric and chemical methods produce similar estimates of total dietary fiber for most foods. However, there are method-dependent variabilities in estimates of soluble and insoluble fiber and in fiber estimates for foods that contain appreciable amounts of lignin or resistant starch.

12.6 STUDY QUESTIONS

1. Define the term *dietary fiber*, and give the constituents that compose dietary fiber.
2. Below are four values for the fiber content (percent dwb) of wheat bran. Using your knowledge of methods to measure crude fiber,acid detergent fiber, neutral detergent fiber (with amylase), and the AOAC method for total dietary fiber, indicate which method most likely fits with each of the four values below. Justify your answer by listing the constituents measured by each method.

Percent Fiber	Methods	Constituents Measured	Procedure
46.0			
8.9			
40.2			
11.9			

3. You are teaching your technician to use the AOAC method for total dietary fiber to determine the dietary fiber content of a new line of high-fiber snack foods being developed by your company. Explain to him or her the purpose(s) of the steps in the total dietary fiber procedure listed below:
   a. heating sample and treating with amyloglucosidase
   b. treating sample with protease
   c. adding four volumes of 95% ethanol to sample after treatment with amyloglucosidase and protease
   d. after drying and weighing the filtered and washed residue, heating one duplicate final product to 525°C in a muffle furnace and analyzing the other duplicate sample for protein.
4. Compare and contrast the AOAC method and the Englyst–Cummings method for determination of total dietary fiber. Consider the principles, procedures, applications, and advantages and disadvantages.
5. Explain how both gravimetric and chemical methods allow differentiation of soluble, insoluble, and total dietary fiber.
6. What is the physiological definition and the chemical nature of resistant starch? What types of foods have relatively high levels of resistant starch? For such foods, will the AOAC procedure or the Englyst–Cummings procedure give you higher fiber values?

12.7 PRACTICE PROBLEMS

1. The following tabular data were obtained when a high-fiber cookie was analyzed for fiber content by the modified AOAC Method 991.43.

	Sample			
	Insoluble		Soluble	
Sample wt. mg	1002.1	1005.3		
Crucible + Celite wt. mg	31,637.2	32,173.9	32,377.5	33,216.4
Crucible + Celite + residue wt. mg	31,723.5	32,271.2	32,421.6	33,255.3
Protein, mg	6.5		3.9	
Crucible + Celite ash wt. mg	32,195.2		33,231.0	

	Blank			
	Insoluble		Soluble	
Crucible + Celite wt, mg	31,563.6	32,198.7	33,019.6	31,981.2
Crucible + Celite + residue wt, mg	31,578.2	32,213.2	33,033.4	33,995.6
Protein, mg	3.2		3.3	
Crucible + Celite + ash wt, mg	32,206.8		31,989.1	

What is the: (a) total, (b) insoluble, and (c) soluble fiber content of the cookie?
2. The amount of dietary fiber in a navy bean cultivar was determined by the Englyst–Cummings procedure. The following data were obtained:

	Sample a	Sample b
Sample weight, mg	195.10	191.30
Neutral sugars, mg	26.36	25.74
Uronic acids, mg	8.74	8.64

How much dietary fiber is in the beans?
3. The following data were obtained when an extruded breakfast cereal was analyzed for total fiber by the AOAC method:

Sample wt (mg)	1002.8
Residue wt (mg)	151.9
Protein wt (mg)	13.1
Ash wt (mg)	21.1
Blank wt (mg)	6.1
Resistant starch (mg)	35.9

What is total fiber % (a) without and (b) with correction for resistant starch?

Answers

1. a = 8.06%; b = 6.06%; c = 2.00%.
2. 17.98%.
3. a = 11.12%; b = 7.54%.

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Crude Fat Analysis

David B. Min and Donald F. Steenson

13.1 INTRODUCTION

13.1.1 Definitions

With proteins and carbohydrates, lipids constitute the principal structural components of foods. Lipids are a group of substances that, in general, are soluble in ether, chloroform, or other organic solvents but are sparingly soluble in water. This solubility characteristic, rather than being a common structural feature of all macromolecules, is unique to lipids (1). Lipids comprise a broad group of substances that have some common properties and compositional similarities (2). However, some lipids, such as triacylglycerols, are very hydrophobic. Others, such as di- and monoacylglycerols, have both hydrophobic and hydrophilic moieties in their molecules and are soluble in relatively polar solvents (1). Triacylglycerols are fats and oils that represent the most prevalent category of a series of compounds known as lipids in foods. The terms lipids, fats, and oils are sometimes used interchangeably.

13.1.2 General Classification

The general classification of lipids that follows is useful to differentiate the lipids in foods (2).

13.1.2.1 Simple Lipids

Ester of fatty acids with alcohol:

- Fats: Esters of fatty acids with glycerol—triacylglycerols
- Waxes: Esters of fatty acids with long-chain alcohols other than glycerols—for example, myricyl palmitate, cetyl palmitate, Vitamin A esters, and Vitamin D esters

13.1.2.2 Compound Lipids

Compounds containing groups in addition to an ester of a fatty acid with an alcohol:

- Phospholipids: Glycerol esters of fatty acids, phosphoric acids, and other groups containing nitrogen—for example, phosphatidyl choline, phosphatidyl serine, phosphatidyl ethanolamine, and phosphatidyl inositol
- Cerebrosides: Compounds containing fatty acids, a carbohydrate, and a nitrogen moiety—such as galactocerebroside and glucocerebroside.
- Sphingolipids: Compounds containing fatty acids, a nitrogen moiety, and phosphoryl group—for example, sphingomyelins

13.1.2.3 Derived Lipids

Derived lipids are substances derived from neutral lipids or compound lipids. They have the general properties of lipids—examples are fatty acids, long-chain alcohols, sterols, fat-soluble vitamins, and hydrocarbons.

13.1.3 Content of Lipids in Foods

The lipid content in bovine milk is shown in Table 13-1. It shows the complexity of milk lipids that differ in polarity and concentrations.

Foods may contain any or all lipid compounds, but those of greatest importance are the triacylglycerols and the phospholipids. Liquid triacylglycerols at room temperature are referred to as oils, such as soybean oil and olive oil, and are generally of plant origin. Solid triacylglycerols at room temperature are termed fats. Lard and tallow are examples of fats, which are generally from animals. The term *fat* is applicable to all triacylglycerols whether they are normally solid or liquid at ambient temperatures. Table 13-2 shows the wide range of lipid content in different foods.

13.1.4 Importance of Analysis

An accurate and precise quantitative analysis of lipids in foods is important for nutritional labeling, to determine whether the food meets the standard of identity and is uniform, and to understand the effects of fats and oils on the functional and nutritional properties of foods.

13-1

table Lipids of Bovine Milk

Kinds of Lipids	Percent of Total Lipids
Triacylglycerols	97–99
Diacylglycerols	0.28–0.59
Monoacylglycerols	0.016–0.038
Phospholipids	0.2–1.0
Sterols	0.25–0.40
Squalene	Trace
Free fatty acids	0.10–0.44
Waxes	Trace
Vitamin A	7–8.5 µg/g
Carotenoids	8–10 µg/g
Vitamin D	Trace
Vitamin E	2–5 µg/g
Vitamin K	Trace

Adapted from (3) with permission of S. Patton, and (4) *Principles of Dairy Chemistry* Jenness, R., and Patton, S. Copyright © 1959. John Wiley & Sons. Inc. with permission.

13-2 Table   Fat Content of Selected Foods

Food Item	Percent Fat (wet weight basis)
Cereals, bread, and pasta	
Rice, white, long-grain, regular, raw, enriched	0.7
Sorghum	3.3
Wheat, soft white	2.0
Rye	2.5
Wheat germ, crude	9.7
Rye bread	3.3
Cracked-wheat bread	3.9
Macaroni, dry, enriched	1.6
Diary products	
Milk, whole, fluid	3.3
Skim milk, fluid	0.2
Cheddar cheese	33.1
Yogurt, plain, whole milk	3.2
Fats and oils	
Lard, shortening, oils	100.0
Butter, with salt	81.1
Margarine, regular, hard, soybean	80.5
Salad dressing	
Italian, commercial, regular	48.3
Thousand Island, commercial, regular	35.7
French, commercial, regular	41.0
Mayonnaise, soybean oil, with salt	79.4
Fruits and vegetables	
Apples, raw, with skin	0.4
Oranges, raw, all commercial varieties	0.1
Blackberries, raw	0.4
Avocados, raw, all commercial varieties	15.3
Asparagus, raw	0.2
Lima beans, inmature seeds, raw	0.8
Sweet corn, yellow, raw	1.2
Legumes	
Soybeans, mature seeds, raw	19.9
Black beans, mature seed, raw	1.4
Meat, poultry, and fish	
Beef, flank, separable lean and fat	10.7
Chicken, broilers or fryers, breast meat only	1.2
Bacon, pork, cured	57.5
Pork, fresh, loin, whole	12.6
Finfish, halibut, Atlantic and Pacific, raw	2.3
Finfish, cod, Atlantic, raw	0.7
Nuts	
Coconut meat, raw	33.5
Almonds, dried, unblanched	52.2
Walnuts, black, dried	56.6
Egg, whole, raw, fresh	10.0

From USDA Nutrient Database for Standard Reference. Release 11-1 (August 1997)
http://www.nal.usda.gov/fnic/cgi-bin/nut_search.pl

13.2 GENERAL CONSIDERATIONS

Lipids are soluble in organic solvents and insoluble in water. Therefore, water insolubility is the essential analytical property used as the basis for the separation of lipids from proteins, water, and carbohydrates in foods. Glycolipids are soluble in alcohols and have a low solubility in hexane. In contrast, triacylglycerols are soluble in hexane and petroleum ether, which are nonpolar solvents. The wide range of relative hydrophobicity of different lipids makes the selection of a single universal solvent impossible for lipid extraction of foods. Some lipids in foods are components of complex lipoproteins and liposaccharides; therefore, suc-

cessful extraction requires that bonds between lipids and proteins or carbohydrates be broken so that the lipids can be freed and solubilized in the extracting organic solvents.

13.3 ANALYTICAL METHODS

The total lipid content of a food is commonly determined by organic solvent extraction methods. The accuracy of these methods greatly depends on the solubility of the lipids in the solvent used. The lipid content of a food determined by extraction with one solvent may be quite different from the content determined with another solvent of different polarity. In addition to solvent extraction methods, there are nonsolvent wet extraction methods and several instrumental methods that utilize the physical and chemical properties of lipids in foods for fat content determination.

Many of the methods cited in this chapter are official methods of AOAC International. Refer to these methods and other original references cited for detailed instructions of procedures.

13.3.1 Solvent Extraction Methods

13.3.1.1 Sample Preparation

The validity of the fat analysis of a food depends on proper sampling and preservation of the sample before the analysis (see also Chapter 5). Good sampling, sample preservation, and proper testing procedures are critical factors in food analyses. An ideal sample should be as close as possible in all of its intrinsic properties to the material from which it is taken. However, a sample is considered satisfactory if the properties under investigation correspond to those of the bulk material within the limits of the test (6).

The sample preparation for lipid analysis depends on the type of food and type and nature of lipids in the food (7). The extraction method for lipids in liquid milk is generally different from that for lipids in solid soybeans. To analyze the lipids in foods effectively, a knowledge of the structure, the chemistry, and the occurrence of the principal lipid classes and their constituents is necessary. Therefore, there is no single standard method for the extraction of all kinds of lipids in different foods. For the best results, sample preparation should be carried out under an inert atmosphere of nitrogen at low temperature to minimize chemical reactions such as lipid oxidation.

13.3.1.1.1 Predrying Sample Lipids cannot be effectively extracted with ethyl ether from moist food because the solvent cannot easily penetrate the moist food tissues. The ether, which is hydroscopic, becomes saturated with water and inefficient for lipid extraction. Drying the sample at elevated temperatures is undesirable because some lipids become bound to proteins and carbohydrates, and bound lipids are not easily extracted with organic solvents. Vacuum oven drying at low temperature or lyophilization increases the surface area of the sample for better lipid extraction. Predrying makes the sample easier to grind for better extraction, breaks fat–water emulsions to make fat dissolve easily in the organic solvent, and helps free fat from the tissues of foods (6).

13.3.1.1.2 Particle Size Reduction The extraction efficiency of lipids from dried foods depends on particle size; therefore, good grinding is very important. The classical method of determining fat in oilseeds involves the extraction of the ground seeds with selected solvent after repeated grinding at low temperature to minimize lipid oxidation. For better extraction, the sample and solvent are mixed in a high-speed comminuting device such as a blender. Lipids are extracted with difficulty from soybeans because of the limited porosity of the soybean hull and its sensitivity to dehydrating agents. The lipid extraction from soybeans is easily accomplished if the beans are broken mechanically by grinding.

13.3.1.1.3 Acid Hydrolysis A significant portion of the lipids in foods such as dairy, bread, flour, and animal products is bound to proteins and carbohydrates, and direct extraction with nonpolar solvents is inefficient. Such foods must be prepared for lipid extraction by acid hydrolysis (Table 13-3). Acid hydrolysis can break both covalently and ionically bound lipids into easily extractable lipid forms. The sample is predigested by refluxing for 1 hr with 3 N hydrochloric acid, and ethanol and solid hexametaphosphate are added to facilitate separation of lipids from other components before foods are extracted with solvents (5, 6). For example, the acid hydrolysis of two eggs requires 10 ml

13-3
table

Effect of Acid Digestion on Fat Extraction from Foods

	Percent Fat Acid Hydrolysis	Percent Fat No Acid Hydrolysis
Dried egg	42.39	36.74
Yeast	6.35	3.74
Flour	1.73	1.20
Noodles	3.77–4.84	2.1–3.91
Semolina	1.86–1.93	1.1–1.37

Adapted from (5) (p. 154), with permission.

of HCl and heating in a water bath at 65°C for 15–25 min or until the solution is clear (5).

13.3.1.2 Solvent Selection

Ideal solvents for fat extraction should have a high solvent power for lipids and low or no solvent power for proteins, amino acids, and carbohydrates. They should evaporate readily and leave no residue, have a relatively low boiling point, and be nonflammable and nontoxic in both liquid and vapor states. The ideal solvent should penetrate sample particles readily, be in single-component form to avoid fractionation, and be inexpensive and nonhygroscopic (5, 6). It is difficult to find an ideal fat solvent to meet all of these requirements. Ethyl ether and petroleum ether are the most commonly used solvents, but pentane and hexane also are used to extract oil from soybeans.

Ethyl ether has a boiling point of 34.6°C and is a better solvent for fat than petroleum ether. It is generally expensive compared to other solvents, has a greater danger of explosion and fire hazards, is hygroscopic, and forms peroxides (5). Petroleum ether is the low boiling point fraction of petroleum and is composed mainly of pentane and hexane. It has a boiling point of 35–38°C and is more hydrophobic than ethyl ether. It is selective for more hydrophobic lipids, cheaper, more nonhygroscopic, and less flammable than ethyl ether. The detailed properties of petroleum ether for fat extraction are described in AOAC Method 945.16 (9).

A combination of two or three solvents is frequently used. The solvents should be purified and peroxide free. The proper solvent-to-solute ratio must be used to obtain the best extraction of lipids from foods (6, 8).

13.3.1.3 Continuous Solvent Extraction Methods

13.3.1.3.1 Goldfish Method—Principle and Characteristics For continuous solvent extraction, solvent from a boiling continuously flows over the sample held in a ceramic thimble. Fat content is measured by weight loss of the sample or by weight of fat removed.

The continuous methods give faster, more efficient extraction than semicontinuous extraction methods. However, they may cause channeling that results in incomplete extraction. The Goldfish (as well as the Wiley and Underwriters) tests are examples of continuous lipid extraction methods (5, 6).

13.3.1.3.2 Goldfish Method—Procedure (See Fig. 13-1)

1. Weigh predried porous ceramic extraction thimble. Place vacuum oven dried sample in thimble
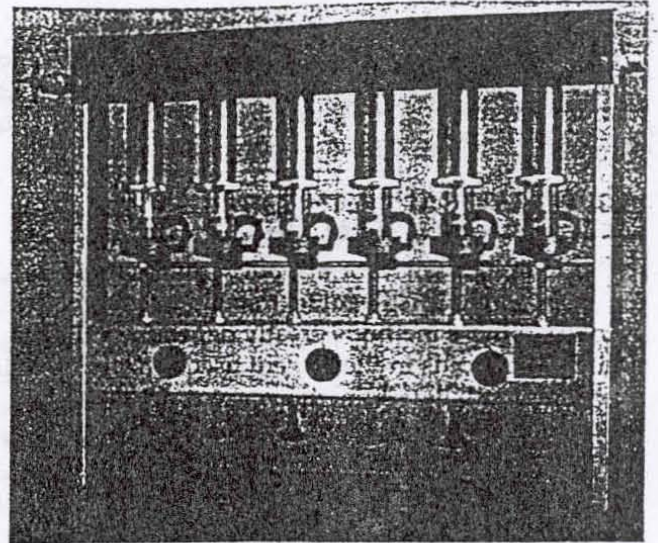
13-1 figure Goldfish fat extractor. (Courtesy of Labconco Corp., Kansas City, MO)

and weigh again. (Sample could instead be combined with sand in thimble and then dried.)
2. Weigh predried extraction beaker.
3. Place ceramic extraction thimble into glass holding tube and then up into condenser of apparatus.
4. Place anhydrous ethyl ether (or petroleum ether) in extraction beaker and put beaker on heater of apparatus.
5. Extract for 4 hr.
6. Lower heater and let sample cool.
7. Remove the extraction beaker and let air dry overnight, then at 100°C for 30 min. Cool beaker in desiccator and weigh.

13.3.1.3.3 Goldfish Method—Calculations

$$\text{Weight of fat in sample} = (\text{beaker} + \text{fat}) - \text{beaker} \quad [1]$$

$$\text{\% Fat on dry weight basis} = (\text{g of fat in sample}/\text{g of dried sample}) \times 100 \quad [2]$$

13.3.1.4 Semicontinuous Solvent Extraction Methods

The Soxhlet method (AOAC Method 920.39C for Cereal Fat; AOAC Method 960.39 for Meat Fat) is an example of the semicontinuous extraction method and is described below.

13.3.1.4.1 Soxhlet Method—Principle and Characteristics For semicontinuous solvent extraction, the solvent builds up in the extraction chamber for 5–10 min

and completely surrounds the sample, then siphons back to the boiling flask. Fat content is measured by weight loss of the sample or by weight of fat removed.

This method provides a soaking effect of the sample and does not cause channeling. However, this method requires more time than the continuous method.

13.3.1.4.2 Soxhlet Method—Preparation of Sample If the sample contains more than 10% H_2O, dry the sample to constant weight at 95–100°C under pressure ≤100 mm Hg for about 5 hr (AOAC Method 934.01).

13.3.1.4.3 Soxhlet Method—Procedure (See Fig. 13-2)

1. Weigh, to the nearest mg, about 2 g of predried sample into a predried extraction thimble, with porosity permitting a rapid flow of ethyl ether. Cover sample in thimble with glass wool.
2. Weigh predried boiling flask.
3. Put anhydrous ether in boiling flask. Note: The anhydrous ether is prepared by washing commercial ethyl ether with two or three portions of H_2O, adding NaOH or KOH, and letting stand until most of H_2O is absorbed from the ether. Add small pieces of metallic Na and let hydrogen evolution cease (AOAC Method 920.39B). Petroleum ether may be used instead of anhydrous ether (AOAC Method 960.39).
4. Assemble boiling flask, Soxhlet flask, and condenser.
5. Extract in a Soxhlet extractor at a rate of 5 or 6 drops per second condensation for about 4 hr, or

Condenser

Siphon Arm —— Thimble
—— Sample

—— Boiling Flask
Solvent —
Heater

13-2
figure

Soxhlet extraction apparatus.

for 16 hr at a rate of 2 or 3 drops per second by heating solvent in boiling flask.

6. Dry boiling flask with extracted fat in an air oven at 100°C for 30 min, cool in desiccator, and weigh.

13.3.1.4.4 Soxhlet Method—Calculation

% Fat on dry weight basis =
(g of fat in sample/g of dried sample) × 100   [3]

13.3.1.5 Discontinuous Solvent Extraction Methods

13.3.1.5.1 Modified Mojonnier Method for Milk Fat— Principle and Characteristics
Fat is extracted with a mixture of ethyl ether and petroleum ether in a Mojonnier flask, and the extracted fat is dried to a constant weight and expressed as percent fat by weight.

The Mojonnier test is an example of the discontinuous solvent extraction method. This extraction method does not require prior removal of moisture from the sample. When including purification of the extracted fat with petroleum ether, this method is very similar to the Roese–Gottlieb Method (AOAC Method 905.02) in both principle and practice.

13.3.1.5.2 Modified Mojonnier Method for Milk Fat (AOAC Method 989.05)—Preparation of Sample
Bring the sample to about 20°C; mix to prepare a homogeneous sample by pouring back and forth between clean beakers. Promptly weigh or measure the test portion. If lumps of cream do not disperse, warm the sample in a water bath to about 38°C and keep mixing until it is homogeneous, using a "policeman" if necessary to reincorporate the cream adhering to the container or stopper. When it can be done without interfering with dispersal of the fat, cool warmed samples to about 20°C before transferring the test portion.

13.3.1.5.3 Modified Mojonnier Method for Milk Fat— Procedure

1. Weigh, to the nearest 0.1 mg, 10 g of milk into a Mojonnier fat extraction flask (Fig. 13-3).
2. Add 1.5 ml of NH_4OH and shake vigorously. Add 2 ml if the sample is sour. NH_4OH neutralizes the acidic sample and dissolves protein.
3. Add 10 ml of 95% ethanol and shake for 90 sec. The alcohol prevents possible gel formation.
4. Add 25 ml of ethyl ether and shake for 90 sec. The ether dissolves the lipid.
5. Cool if necessary, and add 25 ml of petroleum ether and shake for 90 sec. The petroleum ether removes moisture from the ethyl ether extract and dissolves more nonpolar lipid.
6. Centrifuge for 30 sec at 600 rpm.
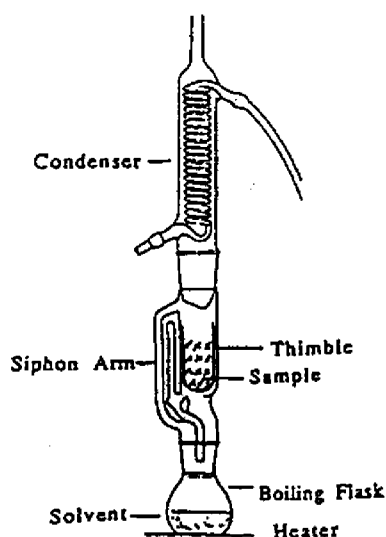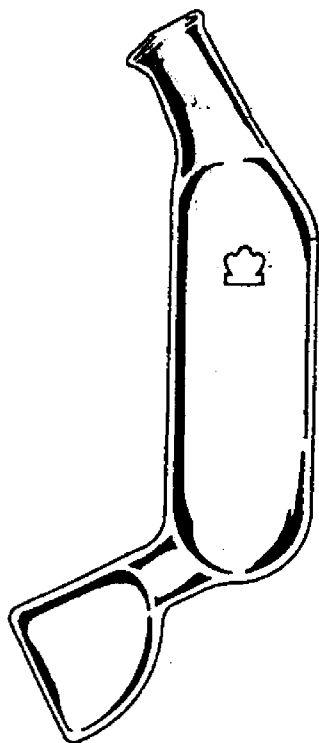
Mojonnier fat extraction flask. (Courtesy of Kontes Glass Co., Vineland NJ)

7. Decant ether solution from the Mojonnier flask into the previously weighed Mojonnier fat dish.

8. Perform second and third extractions in the same manner as for the first extraction described previously (ethanol, ethyl ether, petroleum ether, centrifugation, decant).

9. Evaporate the solvent in the dish on the electric hot plate at ≤100°C in a hood.

10. Dry the dish and fat to a constant weight in a forced air oven at 100°C ± 1°C.

11. Cool the dish to room temperature and weigh.

13.3.1.5.4 Modified Mojonnier Method—Calculations

% Fat = $100 \times \{[(\text{wt dish} + \text{fat}) - (\text{wt dish})]$
$- (\text{avg wt blank residue})]/\text{wt sample}$ [4]

A pair of reagent blanks must be prepared every day. For reagent blank determination, use 10 ml of distilled water instead of milk sample. The reagent blank should be <0.002 g. Duplicate analyses should be <0.03% fat.

13.3.1.5.5 Modified Mojonnier Method for Fat in Flour (AOAC Method 922.06) or Fat in Pet Food (AOAC Method 954.02)
Mix 2 g of sample and 2 ml of ethyl alcohol well in a 50-ml beaker. Add 10 ml of HCl (25 + 11) and place the beaker in a water bath held at 70-80°C

with stirring for 30-40 min for hydrolysis. Add 10 ml of alcohol and cool. The acid hydrolyzed flour is extracted by a combination of ethyl ether and petroleum ether as described in the Modified Mojonnier Method for Milk Fat (AOAC Method 989.05).

13.3.1.6 Elevated Pressure/Temperature Solvent Extraction Methods

Lipid extraction methods involving solvent-sample interactions at high pressure/temperature combinations include supercritical fluid extraction (SFE) and accelerated solvent extraction (ASE). They have gained acceptance recently as substitutes for more traditional solvent extraction methods because of United States Environmental Protection Agency (EPA) regulations that increasingly encourage the reduction of organic solvent use in labs (10).

SFE avoids the use of hazardous organic solvents altogether, thereby completely eliminating the costs and dangers commonly associated with their disposal. Instead, an inert, nontoxic, and inexpensive solvent such as carbon dioxide is heated and pressurized until it displays the properties of a supercritical fluid. In this "hybrid" gas-liquid state, the supercritical fluid's liquid-like density enhances fat extraction from sample matrices, while at the same time its gas-like properties promote separation of the solubilized fat from the solvent fluid after completion of extraction.

While not completely eliminating the use of organic solvents, the ASE process can significantly lower their consumption. Because of the elevated pressures achieved during ASE, fat extraction of samples can take place at temperatures well above the normal boiling point of the solvent used. This enhances solubilization and diffusion of lipids from the sample into the solvent, significantly shortening the extraction time while at the same time reducing solvent consumption.

13.3.1.6.1 Supercritical Fluid Extraction (SFE) Method
1. Principle. A fluid (most often CO_2) is brought to a specific pressure-temperature combination, which allows it to attain supercritical solvent properties for the selective extraction of fat from the sample matrix. The sample is exposed to the supercritical fluid under these controlled conditions of time, temperature, and pressure, allowing dissolution of the fat from the sample. The dissolved fat is then separated from the supercritical solvent by a significant drop in solution pressure. The precipitated fat is then dried and quantified as percent fat by weight.

2. Procedure (See Fig. 13-4)

a. Weigh to the nearest mg, 3-5 g of dry ground sample and place into the extraction cell.
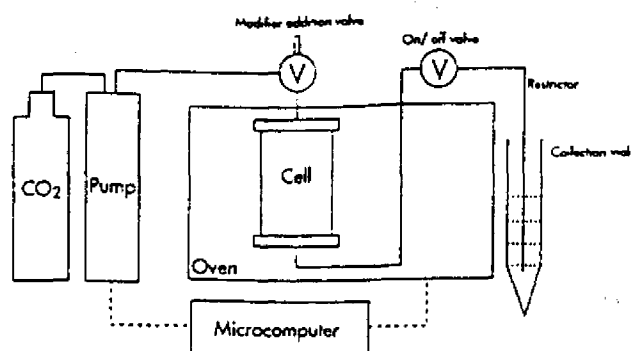
13-4
figure

Experimental setup for supercritical fluid extraction. [From (20), reproduced from the *Journal of Chromatographic Science* by permission of Preston Publications, A Division of Preston Industries, Inc.]

b. Seal the extractor, set oven temperature to 80°C, and pressurize extraction cell to 10,000 psi. The collection vial should be kept at ambient pressure and a temperature of 60–70°C.

c. Using the pump, establish a constant CO_2 flow rate through the extraction cell of 15 standard liters/min.

d. Allow extraction of fat from the extraction cell into the collection vial to continue for 20 min at the proper temperature–pressure settings.

e. Depressurize the extractor, turn off the oven, and remove the collection vial containing extracted fat.

f. Contents of the collection vial can be rotary evaporated (60 min, 50°C, using a preweighed flask) to remove residual CO_2 and/or water from the oil product.

g. Weigh the cooled flask containing the fat product.

3. Calculations.

% Fat = {(wt fat + flask) – wt flask}/wt of
 original dry sample × 100   [5]

4. Applications. Due to the inherent variability in density, chemical composition, etc., among the many substances that can be extracted by SFE, modification of the temperatures and pressures described in the procedure may be necessary to obtain maximal extraction efficiency.

In addition to being used for total fat quantitation (11–14), the pressure–temperature–time variables in SFE can be manipulated to allow selective extraction of minute quantities of polar [15–18] or nonpolar (19) analyses from sample matrices. However, modifiers such as methanol often are necessary to assist supercritical CO_2 in the extraction of highly polar com-

pounds (20). With the proper coupling of the SFE extractor to a chromatographic system such as capillary gas chromatography (GC) (SFE-GC), rapid, accurate separation and quantitation of each extracted analyte is possible (21). Because SFE can save both time and money compared to traditional extraction methods, while at the same time matching their overall precision and accuracy, further applications of this technique in the area of lipid extraction are sure to be developed.

13.3.1.6.2 Accelerated Solvent Extraction (ASE) Method

1. Principle. Fat is extracted by exposing a solid or semisolid sample to a nonpolar solvent in the static or dynamic mode under elevated temperature and pressure conditions. In the static mode, extraction occurs with no outflow of solvent, while the dynamic mode allows fresh solvent to flow continuously through the sample during extraction. Dissolved fat diffuses from the core to the surface of the sample particles, where it is transferred into the extraction solvent. Compressed gas then purges the solubilized fat into a collection vessel. Evaporation separates the solvent from the extracted fat, which is then dry weighed and quantified as percent fat by weight.

2. Procedure (See Fig. 13-5)

a. Weigh, to the nearest mg, 3–5 g of dry ground sample and place into the extraction cell.

b. Tighten the extraction cell in the oven and heat to the extraction temperature of 150°C (temperatures up to 200°C may be used). During the preheating process, open the static valve.

c. After the desired temperature is achieved, open the pump valve and allow solvent to flow into the extraction cell until about 1 ml has accumulated in the collection vial.
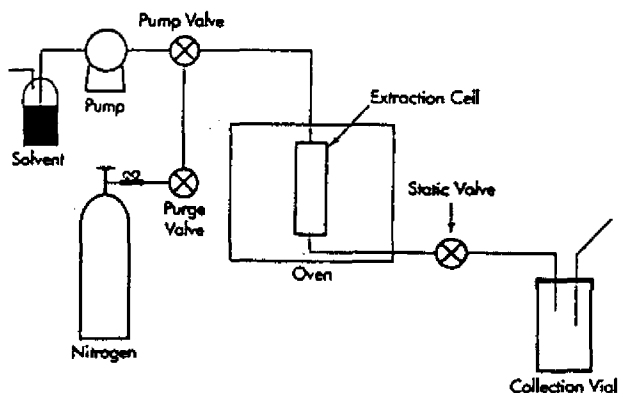
13-5
figure

Experimental setup for accelerated solvent extraction. [Reprinted with permission from (23), Copyright 1996, American Chemical Society.

d. Close the static valve and allow the extraction cell to pressurize to approximately 2000 psi.

e. Allow the pressurized static solvent extraction to proceed for 10 min.

f. Flush the extraction cell with fresh solvent by reopening the static valve, and collect the solvent extract in the vial.

g. Close the pump valve and turn on the purge valve to clear the remaining solvent out of the extraction cell and into the collection vial.

h. Contents of the collection vial can be rotary evaporated (60 min, 50°C, using a preweighed flask) to separate the solvent from the fat product.

i. Weigh the cooled flask containing the fat product.

3. Calculations

% Fat = {(wt fat + flask) − wt flask}/wt of
original dry sample × 100   [6]

4. Applications. Dynamic ASE alone yields the fastest extractions, but also causes increased solvent consumption. Static ASE alone necessitates significantly longer extraction times but minimizes solvent use, making it more appropriate for trace analysis. Combining the static and dynamic modes (as the above procedure does) generally gives the best overall results for typical fat extractions (22).

ASE can provide lipid extractions that are comparable to those obtained by the Soxhlet method and other similar techniques, while using only a fraction of the time and organic solvent. A formal methodology has been proposed as Method 3545 in Update III of the U.S. EPA SW-846 Methods (23, 24).

13.3.2 Nonsolvent Wet Extraction Methods

13.3.2.1 Babcock Method for Milk Fat (AOAC Method 989.04 and 989.10)

13.3.2.1.1 Principle In the Babcock method, H_2SO_4 is added to a known amount of milk in the Babcock bottle. The sulfuric acid digests protein, generates heat, and releases the fat. Centrifugation and hot water addition isolate fat for quantitation in the graduated portion of the test bottle. The fat is measured volumetrically, but the result is expressed as percent fat by weight.

13.3.2.1.2 Procedure (See Fig. 13-6)

1. Accurately pipette the milk sample (17.6 ml) into a Babcock test bottle.

2. Add reagent grade (1.82 sp gr) sulfuric acid (17.5 ml) to the bottle, allowing the acid to flow gently down the neck of the bottle as it is being slowly rotated. The acid digests proteins to liberate the fat.

3. Centrifuge the mixture for 5 min and liquid fat will rise into the calibrated bottle neck. The centrifuge must be kept at 55–60°C during centrifugation.

4. Add hot water to bring liquid fat up into the graduated neck of the Babcock bottle.

5. The direct percentage of fat by weight is read to the nearest 0.05% from the graduation mark of the bottle.

13.3.2.1.3 Applications The Babcock method, which is a most common method for the determination of fat in milk, takes about 45 min and duplicate tests should agree within 0.1%. The Babcock method does not determine the phospholipids in the milk products (26). It is not applicable to products containing chocolate or added sugar without modification because of charring of chocolate and sugars by sulfuric acid. A modified Babcock method is used to determine essential oil in flavor extracts (AOAC Method 932.11) and fat in seafood (AOAC Method 964.12).

13.3.2.2 Gerber Method for Milk Fat

13.3.2.2.1 Principle The principle of the Gerber method (25) is similar to that of the Babcock method, but it uses sulfuric acid and amyl alcohol. The sulfuric acid digests proteins and carbohydrates, releases fat, and maintains the fat in a liquid state by generating heat.

13.3.2.2.2 Procedure

1. Transfer 10 ml of H_2SO_4 at 15–21°C into a Gerber milk bottle.

2. Accurately measure milk sample (11 ml) into the Garter bottle, using a Gerber Pipette.

3. Add 1 ml of isoamyl alcohol to the bottle.

4. Tighten the stopper and mix by shaking the bottle.

5. Centrifuge the bottle for 4 min.

6. Place the bottle in a water bath at 60–63°C for 5 min and then read the fat content from the graduations on the bottle neck.

13.3.2.2.3 Applications The Gerber method is comparable to the Babcock method but is simpler and faster and has wider application to a variety of dairy products. The isoamyl alcohol generally prevents the charring of sugar found with the regular Babcock method.
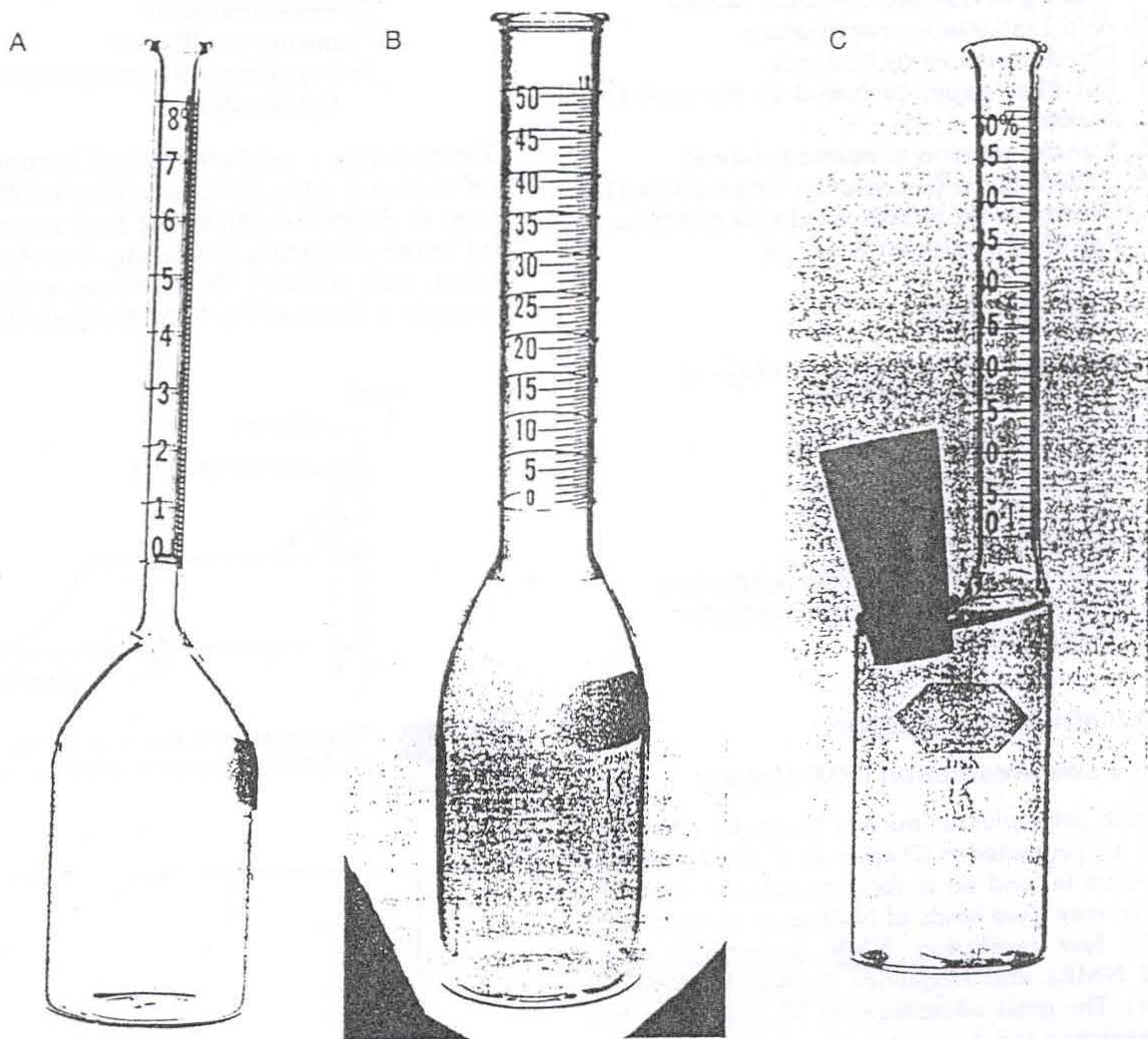
13-6 figure Babcock bottles for milk (A) cream (B), and cheese (Paley bottle) (C) testing. (Courtesy of Kimble Glass Co., Vineland, NJ)

The test is more popular in Europe than in America (27).

13.3.2.3 Detergent Method

The principle of the detergent method is that the detergents react with protein to form a protein–detergent complex to break up emulsions and release fat. The method was originally developed to determine fat in milk because of the corrosive properties of H_2SO_4 in the Babcock test (28). This method was later modified for use with other products. Milk is pipetted into a Babcock test bottle. An anionic detergent, dioctyl sodium phosphate, is added to disperse the protein layer that stabilizes the fat to liberate fat. Then a strong hydrophilic nonionic polyoxyethylene detergent, sorbitan monolaurate, is added to separate fat from other

food components. The percent fat is measured volumetrically and expressed as percent fat (6).

13.3.2.4 Refractive Index Method for Processed Meat

13.3.2.4.1 Principle The refractive index is characteristic of each kind of fat, and the values vary with degree and type of unsaturation, oxidation, heat treatment, temperatures of analyses, and the fat content. Fat is extracted with a solvent, and the refractive index of the solvent is compared to the refractive indices of the extracted fat solution and fat (5, 6).

13.3.2.4.2 Procedure
1. Weigh a 2-g sample.
2. Transfer the sample to mortar and pestle.

3. Add 1.5 g of dry sand.
4. Add 3 g of anhydrous sodium sulfate.
5. Add 3 ml of bromonaphthalene.
6. Grind the materials for 3 min.
7. Put filter paper in funnel in the rack above beaker.
8. Transfer contents of mortar to funnel.
9. Collect filtrate (i.e., solution with extracted fat).
11. Determine the refractive of index of filtrate.
12. Calculate percent fat in sample.

13.3.2.4.3 Calculations

$$\% \text{ Fat} = 100\, V\, d\, (n_1 - n_2)/W\, (n_2 - n) \qquad [7]$$

where:

V = ml of bromonaphthalene
d = density of fat
n = refractive index of fat
n_1 = refractive index of bromonaphthalene
n_2 = refractive index of extracted solution
W = weight of sample

13.3.3 Instrumental Methods

13.3.3.1 Low-Resolution NMR Method

The basic principles of nuclear magnetic resonance (NMR) are presented in Chapter 30. NMR can be used to measure fat and oil in food materials in a nondestructive way. Two kinds of NMR may be used: time domain low resolution NMR (sometimes called pulsed NMR), and frequency domain NMR (NMR spectra). The great advantages of NMR are that it is nondestructive and does not require that the sample be transparent.

In time domain NMR, signals from the hydrogen nuclei (^{1}H or protons) of different food components are distinguished by their different rates of decay or nuclear relaxation. Hydrogen nuclei in solid phases relax extremely fast (signal disappears), while protons in liquid phases relax very slowly. Furthermore, in samples such as oilseeds and some food products, water protons may relax faster than oil protons. The intensity of the signal is proportional to the number of hydrogen nuclei and therefore to hydrogen content. Thus, intensity can be converted to oil content using calibration methods (29–31). This method is used for water content, oil content, solid fat content, and solid-to-liquid ratio.

The equation for the so-called direct method to determine solid fat content follows:

$$S_{dir}(t) = \frac{fs'}{I + fs'} \times 100 \qquad [8]$$

where:

$S_{dir}(t)$ = solid fat content
f = correction factor
s' = intensity at 10 μsec
I = intensity at 70 μsec (corresponding to liquid only)

The intensities s' and I are obtained from the NMR signal as shown in Fig. 13-7. Time domain NMR has been used to analyze fat-containing food materials including butter, margarine, shortening, chocolates, oilseeds, meats, milk powders, cheese, flours, and the like. An example is shown in Fig. 13-8. Here only the liquid sig-
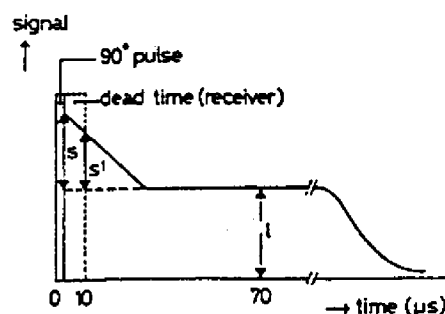
13-7 figure Magnetization decay of a partly crystallized fat. [From (17), used with permission.]
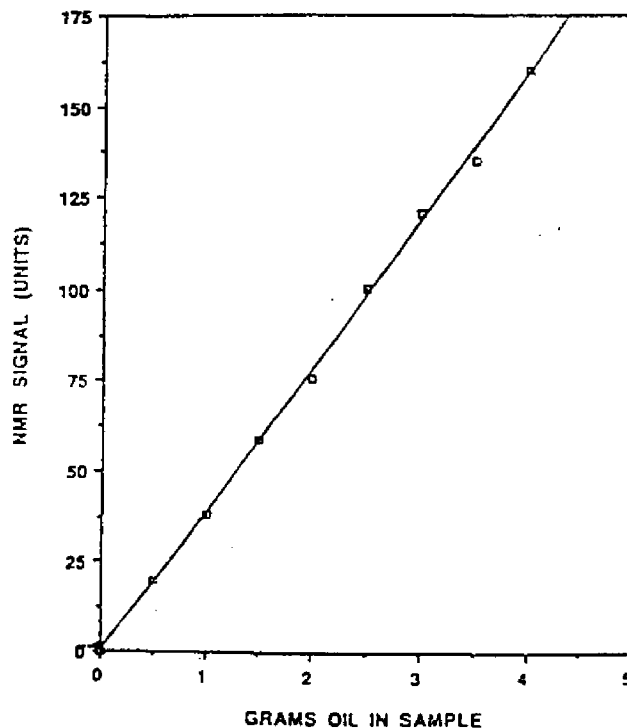
13-8 figure The standard curve for the relationship between wideline NMR signals and oil content of soybean determined by a solvent extraction method. [From (14), used with permission.]

nal intensity was measured. Since liquid oil dominates the liquid-like phases in seeds, a strong correlation of NMR signal with seed oil content (determined in an independent analysis) is obtained. Similar curves have been prepared for dairy foods, meats, and so on. Sometimes it is necessary to remove water by drying the sample before analysis. Alternatively, by taking advantage of the different NMR relaxation times of oil and water, the oil signal can be isolated.

Frequency domain NMR of foods is a new application in which food components are distinguished by the chemical shift (resonance frequency) of their peaks in an NMR spectrum. The pattern of oil resonances reflects degree of unsaturation and other chemical properties. This is useful for chemical analysis because intensities are proportional to amounts. Liquid glycerides have been detected this way in cheeses, fruits, meat, oilseeds, and other food materials. Frequency domain NMR analysis of fats and oils has been reviewed by Eads and Croasmun (33) and by Eads (34).

13.3.3.2 X-Ray Absorption Method

The X-ray absorption of lean meat is higher than that of fat. It has been used for the rapid determination of fat in meat and meat products using the standard curve of the relationship between X-ray absorption and fat content determined by a standard solvent extraction method (6). For example, the AnlyRay fat analysis instrument (BWI Kartridge Pak, Davenport, IA), commonly used to rapidly determine the lean/fat ratio or percent fat of meat products (usually fresh beef or pork), is based on X-ray asorption.

13.3.3.3 Dielectric Method

The dielectric constants of foods change as the oil contents change. For example, the electrical current of lean meat is 20 times greater than that of fat. The coefficient of correlation for the linear regression between the amounts of induced current and the oil contents of soybeans determined by a standard solvent extraction method was 0.98 (20).

13.3.3.4 Infrared Method

This infrared (IR) method is based on absorption of infrared energy by fat at a wavelength of 5.73 μm. The more the energy absorption at 5.73 μm, the higher the fat content of the sample. Mid-infrared spectroscopy is used in Infrared Milk Analyzers to determine milk fat content (AOAC Method 972.16). Near-infrared spectroscopy has been used to measure the fat content of commodities such as meats, cereals, and oilseeds in the laboratory, and is being adapted for on-line measure-

ment. See Chapter 27 for a discussion of infrared spectroscopy.

13.3.3.5 Ultrasonic Method

The acoustic property of fat is different from that of solid-not-fat. The sound velocity in milk increases or decreases as the fat content increases or decreases above or below a certain critical temperature, making it possible to determine the fat content of milk by measuring the sound velocity [36]. Ultrasound also can be used to assess the fat content of animal carcasses (37).

13.3.3.6 Colorimetric Method

The fat content of milk was determined by measuring the color developed by the reaction between milk fat and hydroxamic acid. The color thus developed was compared to a standard curve of the color intensities and fat contents of samples determined by the Mojonnier method (38).

13.3.3.7 Density Measurement Method

The density and oil content of oilseeds have been found to be correlated with $r = -0.96$. The oil content of oilseeds can be determined by measuring the density of seeds using a linear regression line between the seed density and the fat content determined by a standard solvent extraction method (39).

13.3.3.8 Foss–Let Method

Fat content by the Foss–Let method (Foss North America, Eden Prairie, MN) is determined as a function of the specific gravity of a sample solvent extract. A sample of known weight is extracted for 1.5–2 min in a vibration-reaction chamber with perchloroethylene. The extract is filtered and, using a thermostatically controlled device with digital readout, its specific gravity determined. The reading can then be converted to oil or fat percentage using a conversion chart.

13.3.3.9 Milko-Tester Method

Fat content of milk can be measured with the Milko-Tester, which is based on measuring the turbidity of light scattering caused by fat globules in milk (40). A four-stage homogenizer and the design of the photometer in the instrument reduce the effect of fat globule size on the measurement of turbidity. Samples are heated and diluted with a solution of EDTA at an alkaline pH to disperse the casein molecules and reduce turbidity due to protein.

13.4 COMPARISON OF METHODS

Soxhlet extraction or its modified method is the most common crude fat determination method in foods. However, this method requires a dried sample for the hydroscopic ethyl ether extraction. If the samples are moist or liquid foods, the Mojonnier method is generally applicable to determination of the fat content. The instrumental methods such as IR and NMR are very simple, reproducible, and fast, but are available only for fat determination for specific foods. The application of instrumental methods for fat determination generally requires a standard curve between the signal of the instrument analysis and the fat content obtained by a standard solvent extraction method. However, a rapid instrumental method could be used as a quality control method for fat determination of a specific food.

13.5 SUMMARY

Lipids are generally defined by their solubility characteristics rather than by some common structural feature. Lipids in foods can be classified as simple, compound, or derived. The lipid content of foods varies widely, but quantitation is important because of regulatory requirements, nutritive value, and functional properties. To analyze food for the fat content accurately and precisely, it is essential to have a comprehensive knowledge of the general compositions of the lipids in the foods, the physical and chemical properties of the lipids as well as the foods, and the principles of fat determination. There is no single standard method for the determination of fats in different foods. The validity of any fat analysis depends on proper sampling and preservation of the sample prior to analysis. Predrying of the sample, particle size reduction, and acid hydrolysis prior to analysis also may be necessary. The total lipid content of foods is commonly determined by organic solvent extraction methods, which can be classified as continuous (e.g., Goldfish), semicontinuous (e.g., Soxhlet), discontinuous (e.g., Mojonnier), or elevated pressure-temperature (e.g., Supercritical Fluid Extraction). Nonsolvent wet extraction methods, such as the Babcock, Gerber, detergent, and refractive index methods, are commonly used for certain types of food products. A variety of instrumental methods are also available for fat determination of specific foods. These methods are rapid and so may be useful for quality control but generally require correlation to a standard solvent extraction method.

13.6 STUDY QUESTIONS

1. Itemize the procedures that may be required to prepare a food sample for accurate fat determination by a solvent

extraction method (e.g., Soxhlet method). Explain why each of these procedures may be necessary.
2. To extract the fat from a food sample, you have the choice of using ethyl ether or petroleum ether as the solvent, and you can use either a Soxhlet or a Goldfish apparatus. What combination of solvent and extraction would you choose? Give all the reasons for your choice.
3. Explain and contrast the principles (not procedures) involved in determining the fat content of a food product by the following methods. Indicate for each method the type of sample that would be appropriate for analysis.
   a. Soxhlet
   b. Babcock
   c. refractive index
   d. Mojonnier
   e. detergent
   f. low-resolution NMR
4. Differentiate supercritical fluid extraction and accelerated solvent extraction methods. What are their advantages over traditional solvent extraction methods?

13.7 PRACTICE PROBLEMS

1. To determine the fat content of beef by the refractive index method, 5 ml of bromonaphthalene was used to extract fat from 20 g of beef. The density of fat is 0.9 g/ml, and the refractive indices of beef fat, bromonaphthalene, and the bromonaphthalene beef fat extracted solution are 1.466, 1.658, and 1.529, respectively. Calculate the fat content of the beef.
2. To determine the fat content of a semimoist food by the Soxhlet method, the food was first vacuum oven dried. The moisture content of the product was 25%. The fat in the dried food was determined by the Soxhlet method. The fat content of the dried food was 13.5%. Calculate the fat content of the original semimoist product.
3. The densities of milk fat and milk are 0.9 and 1.032, respectively. The fat content of the milk was 3.55% on a volume basis. Calculate the fat content of milk as percent, wet weight basis.
4. The fat content of 10 g of commercial ice cream was determined by the Mojonnier method. The weights of extracted fat after the second extraction and the third extraction were 1.21 g and 1.24 g, respectively. How much of fat, as a percentage of the total, was extracted during the third extraction?

Answers

1. 46.1%; 2. 9.4%; 3. 3.09%; 4. 0.3%.

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Fat Characterization

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14.1 INTRODUCTION

Methods for characterizing edible lipids, fats, and oils can be separated into two categories: those developed to analyze bulk oils and fats, and those focusing on analysis of foodstuffs and their lipid extracts. In evaluating foodstuffs it is usually necessary to extract the lipids prior to analysis and, if sufficient quantities of lipids are available, some of the methods developed for bulk fats and oils can be utilized.

The methods described in this chapter are divided into four sections. The first are traditional analytical methods for bulk fats and oils, many involving "wet chemistry." Then, two sections discuss methods of measuring lipid oxidation. Some of these methods utilize intact foodstuffs but most require the lipids to be extracted from foodstuffs. Last addressed are methods for the analysis of lipid fractions, including fatty acids, triacylglycerols, and cholesterol.

Numerous methods exist for the characterization of lipids, fats, and oils (1–11). In this chapter are included those methods required for the nutritional labeling of food and others appropriate for an undergraduate food analysis course. Many traditional "wet chemistry" methods have been supplemented or superseded by instrumental methods such as gas chromatography (GC), high performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), and Fourier transform infrared (FTIR) spectroscopy. The understanding of basic concepts derived from traditional methods is valuable in learning more sophisticated instrumental methods.

Many of the methods cited are official methods of the American Oil Chemists' Society (AOCS), AOAC International, or the International Union of Pure and Applied Chemists (IUPAC). The principles, general procedures, and applications are described for the methods. Refer to the specific methods cited in Table 14-1 for detailed information on procedures.

14.1.1 Definitions and Classifications

As explained in Chapter 13, the term lipids refers to a wide range of compounds soluble in organic solvents but only sparingly soluble in water. Chapter 13 also outlines the general classification scheme for lipids. The majority of lipids present in foodstuffs are of the following types: fatty acids; mono-, di-, and triacylglycerols; phospholipids; sterols (including cholesterol); and lipid-soluble pigments and vitamins. The commonly used terms monoglyceride, diglyceride, and triglyceride are synonymous with the proper nomenclature terms monoacylglycerol, diacylglycerol, and triacylglycerol, respectively.

In contrast to lipids, the terms fats and oils often refer to bulk products of commerce, crude or refined,

that have already been extracted from animal products or oilseeds and other plants grown for their lipid content. The term fat signifies extracted lipids that are solid at room temperature, and oil refers to those that are liquid. However, the three terms, lipid, fat, and oil, often are used interchangeably.

In relation to the human diet and food labeling, the term fat (e.g., dietary fat, percent fat, or calories from fat) refers to the lipid components of the foodstuff, in contrast to the carbohydrate and protein components. For nutrition labeling purposes (see Chapter 3), total fat is defined as total lipid fatty acids and is expressed as triglycerides. Saturated fat is defined as the sum (in grams) of all fatty acids without double bonds. The optional category of polyunsaturated fat is defined as cis, cis-methylene-interrupted polyunsaturated fatty acids. Also an optional category (unless certain label claims are made), monounsaturated fat is defined as cis-monounsaturated fatty acids. The requirement that the fatty acids be cis prevents inclusion of fatty acids that contain trans isomers from such terms. Thus, there is a need for analyses to distinguish between the two forms (see section 14.6).

14.1.2 Importance of Analyses

Such issues as the effect of dietary fat on health and food labeling requirements necessitate that food scientists be able to not only measure the total lipid content of a foodstuff but also to characterize it. Health concerns require the measurement of such parameters as cholesterol content, amount of saturated and unsaturated fat, and sometimes the type and amount of individual fatty acids. Measurements of lipid stability impact not only the shelf life of the product but also its safety, since some oxidation products (e.g., malonaldehyde, cholesterol oxides) have toxic properties. Another area of interest is the analysis of oils and fats used in deep-fat frying operations. Finally, the development of food ingredients composed of lipids that are not bioavailable (e.g., sucrose polyesters such as Olestra®) or lipids not contributing the normal nine calories per gram to the diet (e.g., short- and medium-chain triglycerides such as Salatrim® and Caprenin®) accentuates the need to characterize the lipids present in food.

14.1.3 Content in Foods and Typical Values

Commodities containing significant amounts of fats and oils include butter; cheese; imitation dairy products such as margarine; spreads; shortening; frying fats; cooking and salad oils; emulsified dressings; peanut butter; confections; and muscle foods such as meat, poultry, and fish. Tables are available that delineate the total fat content of foods (see Chapter 13) as well as their constituent fatty acids [e.g., Section I of AOCS Official Methods (2)]. Ongoing studies are re-

14-1
table   *Correlation of Selected AOCS (2), AOAC (1), and IUPAC (3) Methods[1]*

Method	AOCS	AOAC	IUPAC
Bulk fats and oils			
Refractive index	Cc 7-25	921.08	2.102
Melting point Capillary-tube melting point	Cc 1-25	920.157	
Slip melting point	Cc 3-25		
	Cc 3b-92		
Dropping point	Cc 18-80		
Wiley melting point	Cc 2-38[2]	920.156	
Smoke, Flash, and Fire Points	Cc 9a-48		
	Cc 9b-55		
Cold test	Cc 11-53	929.08	
Cloud point	Cc 6-25		
Color Lovibond	Cc 13e-92		
spectrophotometric	Cc 13c-50		2.103
Iodine value	Cd 1-25[2]	920.159	2.205
	Cd 1d-92	993.20	
	Cd 1c-85		
Saponification number	Cd 3-25	920.16	2.202
	Cd 3a-94		
Free fatty acids (FFAs)	Ca 5a-40	940.28	
Acid value	Cd 3d-63		2.201
Solid fat index (SFI)	Cd 10-57		2.141
Solid fat content (SFC)	Cd 16b-93		2.150
Consistency	Cc 16-60		
Polar components in frying fats	Cd 20-91	982.27	2.507
Lipid Oxidation—Present Status			
Peroxide value	Cd 8-53	965.33	2.501
	Cd 8b-90		
Anisidine value	Cd 18-90		2.504
Hexanal			
Thiobarbituric acid (TBA) test	Cd 19-90		2.531
Lipid oxidation—oxidative stability			
Schaal oven storage			
Oil stability index (OSI)	Cd 12b-92		
Active oxygen method (AOM)	Cd 12-57[2]		2.506
Oxygen bomb			
Lipid fractions			
Fatty acid composition	Ce 1-62	963.22	2.302
		966.06	
Fatty acid methyl esters (FAMEs)	Ce 2-66	969.33	2.301
Polyunsaturated fatty acids (PUFAs)	Cd 15-78	979.19	2.209
trans isomer fatty acids	Cd 14-95		2.207
Cholesterol (and other sterols)		976.26	2.403

[1]ACCS = American Oil Chemists' Society; AOAC = AOAC International (formerly Association of Official Analytical Chemists); IUPAC = International Union of Pure and Applied Chemists.
[2]Though no longer current, these methods are included for reference because of their previous common use.

fining the quantities in food of saturated and unsaturated fat, *trans* isomers, cholesterol, and other specific parameters.

Because of their usefulness as food ingredients, it sometimes is important to know the physical and chemical characteristics of bulk fats and oils. Definitions and specifications for bulk fats and oils (e.g., soybean oil, corn oil, coconut oil), including values for many of the tests described in this chapter, can be found in Section I of the AOCS Official Methods (2), in

the *Merck Index* (12), and in "Fats and Oils" (13). Table 14-2 gives typical values for several of the tests for some of the common commercial fats and oils. It must be remembered that bulk fats and oils can vary markedly in such parameters due to differences in source, composition, and susceptibility to deterioration.

Foods containing even minor amounts of lipids (e.g., <1%) can have a shelf life limited by lipid oxidation and subsequent rancidity.

14-2
table   Typical Values of Selected Parameters for Fats and Oils

Fat/Oil Source	Refractive Index (40°C)	Melting Point (°C)	Iodine Value	Saponification Value
Beef tallow	1.454–1.458	40–48	40–48	190–199
Butterfat (bovine)	1.453–1.456	28–35	26–42	210–233
Cocoa butter	1.456–1.458	31–35	32–40	192–200
Coconut oil	1.448–1.450	23–26	6–11	248–265
Corn	1.465–1.468		107–128	187–195
Cottonseed	1.458–1.466		100–115	189–198
Lard	1.459–1.461	33–46	53–77	190–202
Menhaden	1.472		148–160	189–193
Olive			75–94	184–196
Palm	1.449–1.455	33–40	50–55	190–209
Palm kernel	1.452–1.488	24–26	14–21	230–254
Peanut	1.460–1.465		86–107	187–196
Rapeseed	1.465–1.467		94–120	168–181
Safflower	1.467–1.470		136–148	186–198
Soybean	1.466–1.470		124–139	189–195
Sunflower	1.467–1.469		118–145	188–194

Compiled from (2), *Physical and Chemical Characteristics of Oils, Fats and Waxes, Section I*, with permission from AOCS Press. Copyright 1996.

14.2 GENERAL CONSIDERATIONS

Various fat extraction solvents and methods are discussed in Chapter 13. For lipid characterization, extraction of fat or oil from foodstuffs can be accomplished by homogenizing with a solvent combination such as hexane–isopropanol (3:2, vol/vol) or chloroform–methanol (2:1, vol/vol). The solvent then can be removed using a rotary evaporator or by evaporation under a stream of nitrogen gas. The use of chloroform–methanol as a lipid solvent is common, but is discouraged because of its toxicity. Lipid oxidation during extraction and testing can be minimized by adding antioxidants [e.g., 10–100 mg of butylated hydroxytoluene (BHT) per liter] to solvents and by taking other precautions such as flushing containers with nitrogen gas and avoiding exposure to heat and light.

Sample preparation is hastened through the use of solid-phase extraction (SPE), which consists of passing the lipid extract through a commercially available prepackaged absorbent (e.g., silica gel) that separates contaminants or various fractions (see Chapter 20, section 20.1.3.5.1 and Chapter 33, section 33.2.2.4). Constituents present in lipid extractions that may present problems in lipid characterization include phosphatides, gossypol, carotenoids, chlorophyll, sterols, tocopherols, Vitamin A, and metals.

Bulk fats and oils, after extraction from their parent source, typically undergo the following refinements: degumming, refining, bleaching, and deodorization. Modifications such as fractionation, winterization, interesterification, and hydrogenation also may be a part of the processing, depending on the commodity being produced. Various methods discussed in this chapter can be used to monitor the refining process.

Changes that lipids undergo during processing and storage include lipolysis, oxidation, and thermal degradation (such as during deep-fat frying operations). These changes are discussed in the following sections on methods.

14.3 METHODS FOR BULK OILS AND FATS

Numerous methods exist to measure the characteristics of fats and oils. Some methods (e.g., titer test) have limited use for edible oils (in contrast to soaps and industrial oils). Other methods may require special apparatuses not commonly available or that have been antiquated by common instrumental procedures [e.g., volatile acid methods (Reichert–Meissl, Polenske, and Kirschner values) has been replaced largely by determination of fatty acid composition using GC]. Methods to determine impurities, including moisture, unsaponifiable material, and insoluble impurities, also are not covered in this chapter.

14.3.1 Sample Preparation

Ensure that samples are visually clear and free of sediment. When required (e.g., iodine value), dry the samples prior to testing (AOAC Method 981.11). Because exposure to heat, light, or air promotes lipid oxidation, avoiding these conditions during sample storage will retard rancidity. Sampling procedures are available for bulk oils and fats (AOCS Method C 1–47; 7).

14.3.2 Refractive Index

14.3.2.1 Principle

The refractive index (RI) of an oil is defined as the ratio of the speed of light in air (technically, a vacuum) to the speed of light in the oil.

14.3.2.2 Procedure

Samples are measured with a refractometer at 20 or 25°C for oils and 40°C for fats, since most fats are liquid at this temperature.

14.3.2.3 Applications

RI is used to control hydrogenation; RI decreases linearly as iodine value decreases. RI also is used as a measure of purity and means of identification, since each substance has a characteristic RI.

14.3.3 Melting Point

14.3.3.1 Principle

Melting point may be defined in various ways, each corresponding to a different residual amount of solid fat. The capillary tube melting point, also known as the complete melting point or clear point, is the temperature at which fat heated at a given rate becomes completely clear and liquid in a one-end closed capillary. The slip melting point is performed similarly to the capillary tube method and measures the temperature at which a column of fat moves in an open capillary when heated. The dropping melting point, or dropping point, is the temperature at which the sample flows through a 0.11-inch hole in a sample cup placed in a specialized furnace. The Wiley melting point measures the temperature at which a 1/8 x 3/8 inch disc of fat, suspended in an alcohol–water mixture of similar density, changes into a sphere.

14.3.3.2 Applications

It appears the predominate method in the United States for measuring melting point is the dropping melting point. The procedure has been automated (Mettler Instrument Corporation) and therefore is not labor intensive. The capillary tube method is less useful for oils and fats (in comparison to pure compounds) since they lack a sharp melting point due to their array of various components. The Slip melting point often is used in Europe whereas the Wiley melting point was preferred previously in the United States; the latter is no longer a current AOCS method. A disadvantage of the Wiley melting point is the subjective determination

as to when the disc is spherical. A disadvantage of the Slip melting point is its 16 hr stabilization time.

14.3.4 Smoke, Flash, and Fire Points

14.3.4.1 Principle

The smoke point is the temperature at which the sample begins to smoke when tested under specified conditions. The flash point is the temperature at which a flash appears at any point on the surface of the sample; volatile gaseous products of combustion are produced rapidly enough to permit ignition. The fire point is the temperature at which evolution of volatiles (by decomposition of sample) proceeds with enough speed to support continuous combustion.

14.3.4.2 Procedure

A cup is filled with oil or melted fat and heated in a well-lighted container. The smoke point is the temperature at which a thin, continuous stream of bluish smoke is given off. The flash point and fire point are obtained with continued heating, during which a test flame is passed over the sample at 5°C intervals. For fats and oils that flash at temperatures below 149°C, a closed cup is used.

14.3.4.3 Applications

These tests reflect the volatile organic material in oils and fats, especially free fatty acids and residual extraction solvents. Frying oils and refined oils should have smoke points above 200 and 300°C, respectively.

14.3.5 Cold Test

14.3.5.1 Principle

The cold test is a measure of the resistance of an oil to crystallization. Absence of crystals or turbidity indicates proper winterizing.

14.3.5.2 Procedure

Oil is stored in an ice bath (0 °C) for 5.5 hr and observed for crystallization.

14.3.5.3 Applications

The cold test is a measure of success of the winterizing process. It ensures that oils remain clear even when stored at refrigerated temperatures.

14.3.6 Cloud Point

14.3.6.1 Principle

The cloud point is the temperature at which a cloud is formed in a liquid fat due to the beginning of crystallization.

14.3.6.2 Procedure

The sample is heated to 130°C and then cooled with agitation. The temperature of first crystallization is taken to be the point at which a thermometer in the fat is no longer visible.

14.3.7 Color

Two methods for measuring the color of fats and oils are the Lovibond method and the spectrophotometric method.

14.3.7.1 Procedure

In the Lovibond method, oil is placed in a standard sized tube and visually compared with red and yellow color standards. Results then can be expressed as the number on the standard tube. Automated colorimeters are available.

For the spectrophotometric method, the sample is brought to 25–30°C, placed in a cuvette, and absorbance read at the following wavelengths: 460, 550, 620, and 670 nm. The photometric color index is calculated as shown in Equation [1].

$$\text{Photometric color index} =$$
$$1.29\,(A_{460}) + 69.7(A_{550}) + 41.2(A_{620}) - 56.4(A_{670}) \quad [1]$$

14.3.7.2 Applications

The color of fats and oils is most commonly evaluated using the Lovibond method. Oils and fats from different sources vary in color. But if a refined oil is darker than expected, it is indicative of improper refinement or abuse (13).

Though specifically developed for testing the color of cottonseed, soybean, and peanut oils, the spectrophotometric method is probably applicable to other fats and oils as well.

14.3.8 Iodine Value

14.3.8.1 Principle

The iodine value (or iodine number) is a measure of degree of unsaturation, i.e., the number of carbon–carbon double bonds in relation to the amount of fat or oil. Iodine value is defined as the grams of iodine absorbed per 100-g sample. The higher the amount of unsaturation, the more iodine is absorbed; therefore, the higher the iodine value, the greater the degree of unsaturation.

A common practice is to determine calculated iodine value from the fatty acid composition (see section 14.6.1) using AOCS Recommended Practice Cd 1c-

85. The calculated iodine value is not meant to be a rapid method, but instead gives two results (iodine value of triacylglycerols and free fatty acids) from one analysis (fatty acid composition).

14.3.8.2 Procedure

A quantity of fat or oil dissolved in solvent is reacted with a measured amount of iodine or some other halogen. Halogen addition to double bonds takes place (Equation [2]). A solution of potassium iodide is added to reduce excess ICl to free iodine (Equation [3]). The liberated iodine is then titrated with a standardized solution of sodium thiosulfate using a starch indicator (Equation [4]), and the iodine value is calculated (Equation [5]).

$$ICl + R\text{—}CH\text{=}CH\text{—}R \rightarrow R\text{—}CHI\text{—}CHCl\text{—}R + ICl$$
(excess)   (remaining)   [2]

$$ICl + 2KI \rightarrow KCl + KI + I_2 \quad [3]$$

$$I_2 + \text{starch} + 2Na_2S_2O_3 \rightarrow 2NaI + \text{starch} + Na_2S_4O_6$$
(blue)   (colorless)   [4]

$$\text{Iodine value} = \frac{(B - S) \times N \times 12.69}{\text{sample wt (g)}} \quad [5]$$

where:

B = blank titration, ml
S = sample titration, ml
N = normality of $Na_2S_2O_3$ solution
12.69 is used to convert from mEq thiosulfate to g iodine; molecular weight of iodine is 126.9.

Calculated iodine value is obtained from fatty acid composition using Equation [6] for triacylglycerols. A similar equation allows calculation of the iodine value of free fatty acids.

Iodine value (triglycerides) =
(% hexadecenoic acid × 0.950)
+ (% octadecenoic acid × 0.860)
+ (% octadecadienoic acid × 1.732)
+ (% octadecatrienoic acid × 2.616)
+ (% eicosenoic acid × 0.785)
+ (% docosenoic acid × 0.723)   [6]

14.3.8.3 Applications

Iodine value is used to characterize oils, to follow the hydrogenation process in refining, and as an indication of lipid oxidation, since there is a decline in unsaturation during oxidation.

The calculated value tends to be low for materials with a low iodine value and for oils with greater than 0.5% unsaponifiable material (e.g., fish oils).

14.3.9 Saponification Value

14.3.9.1 Principle

Saponification is the process of breaking down or degrading a neutral fat into glycerol and fatty acids by treatment of the fat with alkali (Equation [7]).

$$
\begin{array}{ccc}
\text{triacylglycerol} & \text{glycerol} & \text{potassium salt of fatty acids}
\end{array}
$$

[7]

The saponification value (or saponification number) is defined as the amount of alkali necessary to saponify a given quantity of fat or oil. It is expressed as the milligrams of KOH required to saponify 1 g of the sample. The saponification value is an index of the mean molecular weight of the triacylglycerols in the sample. The mean molecular weight of the triacylglycerols may be divided by 3 to give an approximate mean molecular weight for the fatty acids present. The smaller the saponification value, the longer the average fatty acid chain length.

In common practice, the calculated saponification value is determined from the fatty acid composition (see section 14.6.1) using AOCS Recommended Practice Cd 3a-94.

14.3.9.2 Procedure

Excess alcoholic potassium hydroxide is added to the sample and the solution is heated to saponify the fat (Equation [7]). The unreacted potassium hydroxide is back-titrated with standardized HCl using phenolphthalein as the indicator and the saponification value is calculated (Equation [8]).

$$
\text{Saponification value} = \frac{(B - S) \times N \times 56.1}{\text{sample wt (g)}} \quad [8]
$$

where:

B = blank titration, ml
S = sample titration, ml
N = normality of the HCl
56.1 = molecular weight of KOH

The calculated saponification value is obtained from fatty acid composition using Equation [9]. The

fractional molecular weight of each fatty acid in the sample must be determined first by multiplying the fatty acid percentage (divided by 100) by its molecular weight. The mean molecular weight is the sum of the fractional weights of all the fatty acids in the sample.

$$
\text{Saponification value} = \frac{3 \times 56.1 \times 1000}{[(\text{mean molecular weight} \times 3) + 92.09] - (3 \times 18)} \quad [9]
$$

where:

56.1 = molecular weight of KOH
92.09 = molecular weight of glycerol

14.3.9.3 Applications

The calculated saponification value is not applicable to fats and oils containing high amounts of unsaponifiable material, free fatty acids (>0.1%), or mono-and diacylglycerols (>0.1%).

14.3.10 Free Fatty Acids (FFAs) and Acid Value

14.3.10.1 Principle

Measures of fat acidity normally reflect the amount of fatty acids hydrolyzed from triacylglycerols (Equation [10]).

$$
\begin{array}{ccc}
\text{triacylglycerol} & \text{glycerol} & \text{fatty acids}
\end{array}
$$

[10]
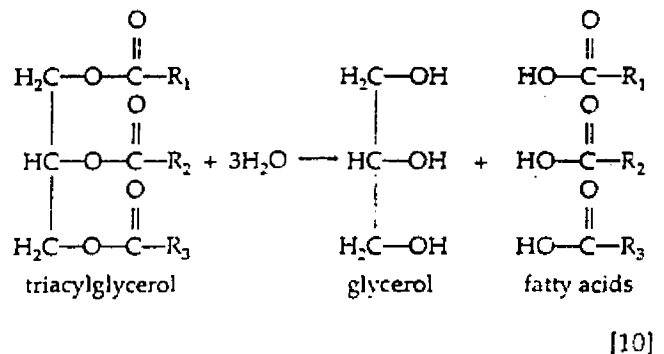
Free fatty acid (FFA) is the percentage by weight of a specified fatty acid (e.g., percent oleic acid). Acid value is defined as the mg of KOH necessary to neutralize the free acids present in 1 g of fat or oil. In addition to free fatty acids, acid phosphates and amino acids also can contribute to acidity. In samples containing no acids other than fatty acids, FFA and acid value may be converted from one to the other using a conversion factor (Equation [11]). Acid value conversion factors for lauric and palmitic are 2.81 and 2.19, respectively.

$$
\% \text{ FFA (as oleic)} \times 1.99 = \text{acid value} \quad [11]
$$

Sometimes the acidity of edible oils and fats is expressed as milliliters of N NaOH required to neutralize the fatty acids in 100 g of fat or oil (9).

14.3.10.2 Procedure

To a liquid fat sample, neutralized 95% ethanol and phenolphthalein indicator are added. The sample then is titrated with NaOH and the percent FFA calculated (Equation [12]).

$$\% \text{ FFA (as oleic)} = \frac{\text{ml alkali} \times N \text{ of alkali} \times 28.2 \text{ mg}}{\text{sample wt}} \quad [12]$$

14.3.10.3 Applications

In crude fat, FFA or acid value estimates the amount of oil that will be lost during refining steps designed to remove fatty acids. In refined fats, a high acidity level means a poorly refined fat or fat breakdown after storage or use. However, if a fat seems to have a high amount of FFAs, it may be attributable to acidic additives (e.g., citric acid added as a metal chelator) since any acid will participate in the reaction (13). If the fatty acids liberated are volatile, FFA or acid value may be a measure of hydrolytic rancidity.

14.3.11 Solid Fat Index (SFI) and Solid Fat Content (SFC)

14.3.11.1 Principle

Originally, the amount of solids in a fat was estimated using the solid fat index (SFI). SFI is measured using dilatometry, which determines the change in volume with change in temperature. As solid fat melts, it increases in volume. Plotting volume against temperature gives a line at which the fat is solid, a line at which it is liquid, and a melting curve in between (Fig. 14-1).
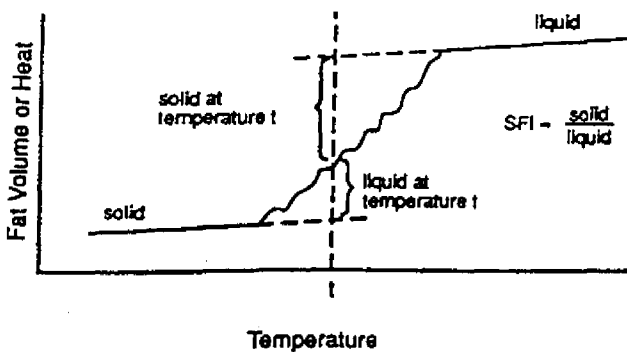
Temperature

Melting curve of a glyceride mixture. [Adapted from (11), p. 249, by courtesy of Marcel Dekker, Inc.]

However, because the solid fat line is difficult to determine experimentally, a line is placed 0.100 specific volume units (ml/g) below the liquid line with the same slope. The SFI is the volume of solid fat divided by the volume between the upper and lower lines, expressed as a percentage (13).

Preferably, the actual percent solid fat in a sample, termed the solid fat content (SFC), can be determined using either continuous wave or pulse NMR (see Chapter 13 and 30).

14.3.11.2 Procedure

For SFI, fat dilatometers consist of a bulb connected to a calibrated capillary tube. As fat in the bulb expands upon heating, it forces a liquid (i.e., colored water or mercury) into the capillary tube.

14.3.11.3 Applications

The amount of solid fat phase present in a plastic fat (e.g., margarine, shortening) depends on the type of fat, its history, and the temperature of measurement. The proportion of solids to liquids in the fat and how quickly the solids melt have an impact on functional properties, such as the mouthfeel of a food.

Chapter 30 explains NMR and gives examples of measuring the solid content of fat and other foods. Though the equipment is expensive, SFC is preferred over SFI because it measures the actual fat content, is less subject to error, and takes less time. Comparison between samples must be made using SFC (or SFI) values taken at the same temperature.

14.3.12 Consistency

Consistency, often described using terms such as plasticity, hardness, creaminess, and spreadability, is an important property of plasticized fats (e.g., shortenings, margarine, butter).

14.3.12.1 Principle

The penetrometer method of determining consistency measures the distance a cone-shaped weight will penetrate a fat in a given time period.

14.3.12.2 Applications

The penetrometer method is useful for measuring the consistency of plastic fats and solid fat emulsions. Like SFI and SFC, consistency is dependent on the type of fat, its history, and the temperature during measurement.

14.3.13 Polar Components In Frying Fats

Methods used to monitor the quality of the oil or fat used in deep-fat frying operations are based on the physical and chemical changes that occur, which include an increase in each of the following parameters: viscosity, foaming, free fatty acids, degree of saturation, hydroxyl and carbonyl group formation, and saponification value. Standard tests used in the evaluation of frying fats include quantitating polar components, conjugated dienoic acids, and fatty acid composition. In addition, there are several rapid tests useful in day-to-day quality assurance of deep-fat frying operations (14).

14.3.13.1 Principle

Deterioration of used frying oils and fats can be monitored by measuring the polar components, which include monoacylglycerols, diacylglycerols, free fatty acids, and other products formed during heating of foodstuffs. Nonpolar compounds are primarily unaltered triacylglycerols. The polar compounds in a sample can be separated from nonpolar compounds using chromatographic techniques.

14.3.13.2 Procedure

Polar components are measured by dissolving the fat sample in light petroleum ether–diethyl ether (87:13), then applying the solution to a silica gel column. Polar compounds are adsorbed onto the column. Nonpolar compounds are eluted, the solvent evaporated, the residue weighted, and the total polar components estimated by difference. Quality of the determination can be verified by eluting polar compounds and separating polar and nonpolar components using thin-layer chromatography.

14.3.13.3 Applications

A suggested limit of 27% polar components in frying oil is a guide for when it should be discarded. A limitation of this method is the sample run time of 3.5 hr (14).

14.4 LIPID OXIDATION—MEASURING PRESENT STATUS

The term rancidity refers to the off odors and flavors resulting from lipolysis (hydrolytic rancidity) or lipid oxidation (oxidative rancidity). Lipolysis is the hydrolysis of fatty acids from the glyceride molecule. Because of their volatility, hydrolysis of short-chained fatty acids can result in off odors.

Lipid oxidation (also called autoxidation) as it

occurs in bulk fats and oils proceeds via a self-sustaining free radical mechanism that produces hydroperoxides (initial or primary products) that undergo scission to form various products including aldehydes, ketones, organic acids, and hydrocarbons (final or secondary products) (15) (see Fig. 14-2).

In biological tissues, including foodstuffs, abstraction reactions and rearrangements of alkoxyl and peroxyl radicals result in the production of endoperoxides and epoxides as secondary products. Many methods have been developed to measure the different compounds as they form or degrade during lipid oxidation. Since the system is dynamic, it is recommended that two or more methods be used to obtain a more complete understanding of lipid oxidation.

Measuring the current status of a fat or oil in regard to lipid oxidation can be achieved using such procedures as peroxide value, anisidine value, hexanal measurement, and the thiobarbituric acid (TBA) test. Some of these procedures have been modified (especially with respect to sample size) for use in biological tissue assays (16). Other methods that monitor lipid oxidation (and that vary in usefulness) include the iodine value, acid value, Kreis test, and oxirane test, as well as the measurement of conjugated dienes and trienes, total and volatile carbonyl compounds, polar compounds, and hydrocarbon gases (6, 11).

While quantitating lipid oxidation by one or more of the methods listed above is usually adequate, in some cases it may be necessary to visualize the location of lipid molecules and lipid oxidation within a food or raw ingredient. Fluorescence microscopy with stains specific to lipids can be applied to such a problem. For example, the dye Nile Blue (with the active ingredient Nile Red) can be combined with a lipid-containing sample and the preparation viewed under a fluorescence microscope (17–19). Lipids will appear an intense yellow fluorescence, with the intensity of the fluorescence changed by the nature of the lipids and by

Time

14-2 Changes in quantities of lipid oxidation reactants and products over time. [Adapted from (15), with permission from Labuza, T.P. Kinetics of lipid oxidation in foods, in Critical Reviews in Food Technology, Vol. 2, Issue 3, Copyright CRC Press, Boca Raton, FL, © 1971.]

lipid oxidation. Example applications include localizing oxidized lipids in a cereal product; visualizing interactions between lipids and emulsifiers; and localizing lipids in cheeses, frosting, and chocolates.

14.4.1 Sample Preparation

Most methods require lipid extraction prior to analysis (see section 14.2). However, variations of some methods (e.g., some TBA tests) begin with the original foodstuff.

14.4.2 Peroxide Value

14.4.2.1 Principle

Peroxide value is defined as the milliequivalents (mEq) of peroxide per kilogram of fat. It is a titrimetric determination of the amount of peroxide or hydroperoxide groups, the initial products of lipid oxidation.

14.4.2.2 Procedure

The fat or oil sample is dissolved in glacial acetic acid–isooctane (3:2). Upon addition of excess potassium iodide which reacts with the peroxides, iodine is liberated (Equation [13]). The solution then is titrated with standardized sodium thiosulfate using a starch indicator (Equation [14]). Peroxide value is calculated as shown in Equation [15].

$$ROOH + K^+ I^- \xrightarrow{\text{H}^+, \text{heat}} ROH + K^+OH^- + I_2 \quad [13]$$
$$\text{(excess)}$$

$$I_2 + \text{starch} + 2Na_2S_2O_3 \rightarrow 2NaI + \text{starch} + Na_2S_4O_6 \quad [14]$$
$$\text{(blue)} \hspace{4cm} \text{(colorless)}$$

$$\text{Peroxide value} = \frac{(S - B) \times N \times 1000}{\text{sample wt (g)}} \quad [15]$$
$$\text{(mEq peroxide / kg fat)}$$

where:

S = sample titration, ml
B = blank titration, ml
N = normality of $Na_2S_2O_3$ solution

14.4.2.3 Applications

Peroxide value measures a transient product of oxidation, i.e., after forming, peroxides break down to form other products. A low value may represent either the beginning of oxidation or advanced oxidation (see Fig. 14-2), which can be distinguished by measuring peroxide value over time. For determination in foodstuffs, a disadvantage of this method is the 5-g fat or oil sample

size required; it is difficult to obtain sufficient quantities from foods low in fat. This method is empirical and any modifications may change results. Despite its drawbacks, peroxide value is one of the most common tests of lipid oxidation.

14.4.3 Anisidine Value and Totox Value

14.4.3.1 Principle

The anisidine value determines the amount of aldehydes (mainly 2-alkenals and 2,4-dienals) in fats and oils. The aldehydes react with p-anisidine to form a chromogen that is measured spectrophotometrically. The totox value tends to indicate the total oxidation of a sample using both the peroxide and anisidine values (Equation [16]).

$$\text{Totox value} = \text{anisidine value} + (2 \times \text{peroxide value}) \quad [16]$$

14.4.3.2 Procedure

The fat or oil is dissolved in isooctane and reacted with p-anisidine. After 10 min the absorbance is measured at 350 nm. Anisidine value is calculated as shown in Equation [17].

$$\text{Anisidine value} = \frac{25 \times (1.2 A_S - A_B)}{\text{sample wt (g)}} \quad [17]$$

where:

A_S = absorbance after reaction
A_B = absorbance before reaction

14.4.3.3 Applications

Since peroxide value measures hydroperoxides (which increase and then decrease) and anisidine value measures aldehydes (decay products of hydroperoxides which continually increase), the totox value usually rises continually during the course of lipid oxidation. Though not common in the United States, anisidine and totox values are extensively used in Europe (13).

14.4.4 Hexanal

14.4.4.1 Principle

Of the various products produced during lipid oxidation, one of the common aldehydes formed is hexanal. Measurement of headspace hexanal is another means of monitoring the extent of lipid oxidation. It is performed commonly using direct headspace analysis, which entails the chromatographic analysis of a set volume of vapor obtained from the headspace above a sample held in a closed container (see Chapter 33, section 33.2.2.1).

14.4.4.2 Procedure

No official method exists for obtaining a measurement of hexanal; a review of research literature will indicate current practices used with various commodities. Typically, a small sample of the commodity is placed in a container having a septum cap. An internal standard may be added, for example, 4-heptanone (20). The container is sealed and then heated for a given time. Heating increases the concentration of headspace volatiles (21). Using a gas syringe, an aliquot of the vapors in the container headspace then is removed and injected into a GC equipped with a flame ionization detector. The quantity of hexanal is then calculated from the peak area (see Chapters 31 and 33).

14.4.4.3 Applications

Hexanal may correlate well with sensory determination of lipid oxidation since it is a major contributor to off flavors in some food commodities. The quantity of other products of lipid oxidation, for example, pentanal, is easily obtained simultaneously with hexanal measurement, and may enhance the characterization of lipid oxidation in various food commodities. An advantage of this method is that lipid extraction is not required. Automated headspace samplers are available that both heat the sample and ensure a constant volume is being analyzed.

14.4.5 Thiobarbituric Acid (TBA) Test

14.4.5.1 Principle

The thiobarbituric acid (TBA) test measures a secondary product of lipid oxidation, malonaldehyde. It involves reaction of malonaldehyde (or malonaldehyde-type products) with TBA to yield a colored compound that is measured spectrophotometrically. Because the reaction is not specific to malonaldehye, results sometimes are reported as TBA reactive substances (TBARS). The food sample may be reacted directly with TBA, but is often distilled to eliminate interfering substances, then the distillate is reacted with TBA. Many modifications of the test have been developed.

14.4.5.2 Procedure

In contrast to the direct method for fats and oils (see Table 14-1), a commonly used procedure (22, 23) is outlined here that requires distillation of the food commodity prior to determining TBA reactive substances. A weighed sample is combined with distilled water and mixed. The pH is adjusted to 1.2 and the sample is transfered to a distillation flask. After addition of BHT

(optional), antifoam reagent, and boiling beads, the sample is distilled rapidly and the first 50 ml collected. An aliquot of the distillate is combined with TBA reagent and heated in a boiling water bath for 35 min. Absorbance of the solution is determined at 530 nm and, using a standard curve, absorbance readings are typically converted to milligrams of malonaldehyde (or TBARS) per kilogram of sample.

14.4.5.3 Applications

The TBA test correlates better with sensory evaluation of rancidity than does peroxide value, but like peroxide value it is a measure of a transient product of oxidation (i.e., malonaldehyde readily reacts with other compounds). Despite its limited specificity and the large sample sizes possibly required (depending on the method), the TBA test with minor modifications is frequently used to measure lipid oxidation, especially in meat products.

An alternative to the spectrophotometric method described is to determine the actual content of malonaldehyde using HPLC analysis of the distillate.

14.5 LIPID OXIDATION—EVALUATING OXIDATIVE STABILITY

Because of their inherent properties (e.g., the amount of unsaturation and the presence of natural antioxidants) as well as external factors (e.g., added antioxidants, processing and storage conditions), lipids and foodstuffs containing lipids vary in their susceptibility to rancidity. The resistance of lipids to oxidation is known as oxidative stability. Inasmuch as determining oxidative stability using actual shelf life determinations at ambient conditions of storage (usually room temperature) requires months or even years, accelerated tests have been developed to evaluate the oxidative stability of bulk oils and fats, and foodstuffs. Accelerated tests artificially hasten lipid oxidation by exposing samples to heat, oxygen, metal catalysts, light, or enzymes. A major problem with accelerated tests is assuming reactions that occur at elevated temperatures or under other artificial conditions are the same as normal reactions occurring at the actual storage temperature of the product. An additional difficulty is ensuring that the apparatus is clean and completely free of metal contaminants and oxidation products from previous runs. Therefore, assuming lipid oxidation is the factor that limits shelf life, shelf life determinations at ambient conditions should accompany and hopefully validate the results of accelerated tests of oxidative stability.

Induction period is defined as the length of time before detectable rancidity, or time before rapid accel-

eration of lipid oxidation (see Fig. 14-2). Induction period can be determined by such methods as calculating the maximum of the second derivative with respect to time or manually drawing tangents to the lines (Fig. 14-3). Measurement of the induction period allows a comparison of the oxidative stability of samples that contain differing ingredients or of samples held at varying storage conditions, and it provides an indication of the effectiveness of various antioxidants in preventing lipid oxidation.

14.5.1 Schaal Oven Storage Protocol

As a means of accelerating the determination of oxidative stability, Schaal oven storage is often used. This protocol consists of placing a fat or oil of known weight in a heated environment at a specified temperature, usually about 60°C. Inasmuch as the temperature, the type of heating, the sample container dimensions and composition, and other such parameters have never been specified, this ill-defined protocol is not an official method. To allow replication by other laboratories, reported research must include such details. However, 60°C is a desirable accelerated storage temperature since the mechanism of oxidation at this temperature is the same as oxidation at room temperature; this is in contrast to the differing mechanisms that occur at more elevated (e.g., 100°C) temperatures (24).

To determine an induction period and thus oxidative stability, the Schaal oven storage protocol must be combined with some method of detecting rancidity, for example, sensory evaluation or peroxide value. Results of oxidative stability determinations obtained at approximately 60°C correlate well with actual shelf life determinations (9).
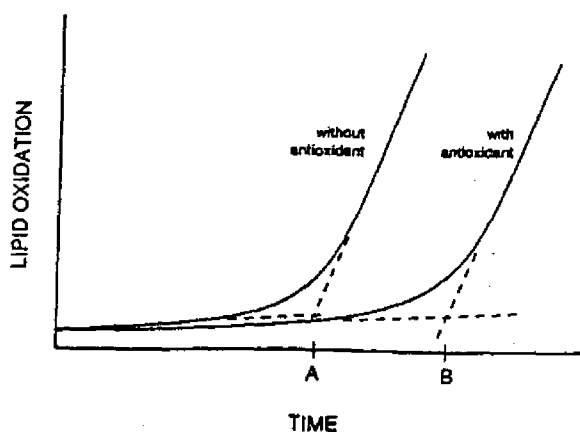
TIME

A plot of lipid oxidation over time, showing the effect of an antioxidant on induction period. Time A is induction period of sample without antioxidant and Time B is induction period of sample with antioxidant.

14.5.2 Oil Stability Index (OSI) and Active Oxygen Method (AOM)

14.5.2.1 Principle

The oil stability index (OSI) determines induction period by bubbling purified air through an oil or fat sample held at an elevated temperature (often 110 or 130°C), then passing the acidic volatiles (primarily formic acid) into a deionized water trap. The conductivity of the water is measured continuously, resulting in data similar to those shown in Fig. 14-3. Results should specify the temperature used as well as induction period time. Two instruments that automate this method are the Rancimat® (Brinkmann Instruments, Inc.) and the Oxidative Stability Instrument® (Omnion, Inc.). The more familiar but outdated and labor intensive active oxygen method (AOM) is similar to the OSI except induction period is determined by discontinuous measurements of either peroxide value or sensory evaluation of rancid odor.

14.5.2.2 Applications

These methods were designed originally to measure the effectiveness of antioxidants. The OSI is determined much faster than tests performed at Schaal oven storage temperatures, but results from the latter may correlate better with actual shelf life (9, 24). Specification sheets for fats and oils often report AOM values, to accommodate individuals working in this area who are familiar with AOM values. OSI values can be converted to AOM values.

Applicable to all fats and oils, the OSI has also been researched for applicability to certain low-moisture snack foods (e.g., potato chips and corn chips). Because of the continuous exposure to circulating air, samples that contain more than negligible amounts of water tend to dehydrate during the determination and are not likely to give reliable results.

14.5.3 Oxygen Bomb

14.5.3.1 Principle

Inasmuch as lipid oxidation results in the uptake of oxygen from the surrounding environment (see Fig. 14-2), measuring the time required for the onset of rapid disappearance of oxygen in a closed system provides a means of determining oxidative stability.

14.5.3.2 Procedure

The oxygen bomb consists of a heavy-walled container that has a pressure recorder attached. The sample is placed in the container and oxygen is used to pressurize the container to 100 psi. The container then is

placed in a boiling water bath. Induction period is determined by measuring the time until a sharp drop in pressure occurs, which corresponds with the rapid absorption of oxygen by the sample.

14.5.3.3 Applications

Oxygen bomb results may have a better correlation with rancidity shelf life tests than AOM values. Another advantage, compared to the OSI (or AOM), is that the oxygen bomb method may be used with intact foodstuffs instead of extracted lipids (13).

14.6 METHODS FOR LIPID FRACTIONS

The lipid present in food commodities or bulk fats and oils can be characterized by measuring the amount of its various fractions, which include fatty acids, mono-, di-, and triacylglycerols, phospholipids, sterols (including cholesterol), and lipid-soluble pigments and vitamins. Another means of categorizing lipid fractions is inherent in nutrition labeling which involves the measurement of not only total fat but also saturated fat, polyunsaturated fat, and monounsaturated fat. In addition, foods may contain lipids that do not contribute the same caloric content as normal lipids, for example, sucrose polyesters (e.g., Olestra®), medium-chain triglycerides, and triglycerides that contain short-chain fatty acids (e.g., Salatrim*, Caprenin®). Many of these fractions are determined readily by evaluating the component fatty acids. From the fatty acid composition, calculations are made to determine such parameters as total fat, saturated fat, calculated iodine value, and calculated saponification value.

In contrast, the measurement of total and saturated fat in foods containing Olestra® requires special consideration. AOAC peer-verified Method PVM 4:1995 outlines the use of lipase on the lipid extract, which yields fatty acids and unreacted Olestra*. The fatty acids are converted to calcium soaps and Olestra* is extracted and discarded. The precipitated soaps are converted back to fatty acids, which are subsequently analyzed via capillary gas chromatography.

Gas chromatography (GC) (see Chapter 33) is ideal for the analysis of lipids. GC can be used for such determinations as total fatty acid composition, distribution and position of fatty acids in lipid, studies of fat stability and oxidation, assaying heat or irradiation damage to lipids, and detection of adulterants and antioxidants (9). Methods exist that detail the analysis of various lipid fractions using GC (5). GC combined with mass spectroscopy (MS) (see Chapter 29) is a powerful tool used in identification of compounds. High performance liquid chromatography (HPLC) (see Chapter 32) also is useful in lipid analyses (25). Thin-layer chromatography (TLC) (see Chapter 31)

has been used extensively in the past by lipid chemists. Partly due to low cost and ease, TLC is still useful, though many assays may be more quantitative or have better resolution using GC or HPLC.

14.6.1 Fatty Acid Composition and Fatty Acid Methyl Esters (FAMEs)

The fatty acid composition, or fatty acid profile, of a food product is determined by quantifying the kind and amount of fatty acids that are present, usually by extracting the lipids and analyzing them using capillary GC.

14.6.1.1 Principle

To increase volatility before GC analysis, triacylglycerols and phospholipids are typically saponified and the fatty acids thus liberated are esterified to form fatty acid methyl esters (FAME) (Equation [18]). Figure 14-4 is a chromatogram showing separation of FAMEs of varying length and unsaturation, as listed in Table 14-3.

[18]
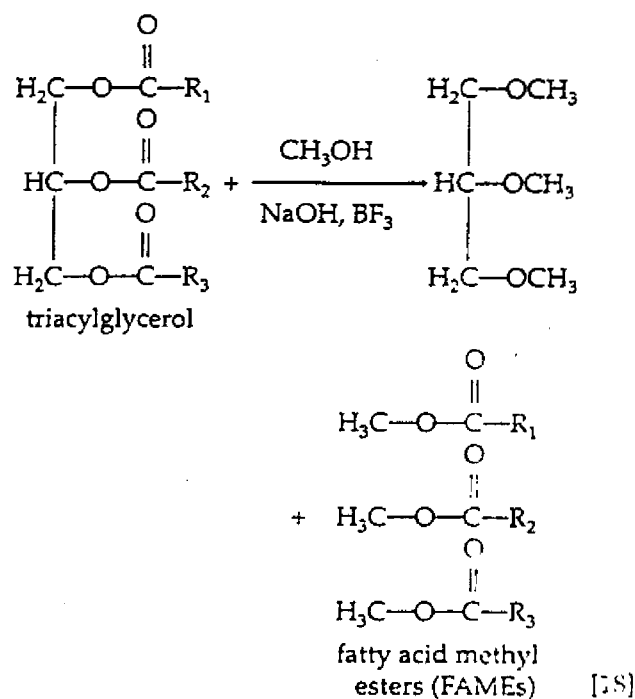
14.6.1.2 Procedure

The lipid is extracted from the food, for example, by homogenizing with a suitable solvent such as hexane–isopropanol (3:2, vol/vol) and then evaporating the solvent. The FAMEs are prepared by combining the extracted lipid with sodium hydroxide, methanol, boron trifluoride, and heptane, and then refluxing. (An alternative to the use of boron trifluoride is sulfuric

Supelco 37 Component FAME Mix on SP-2560 Column
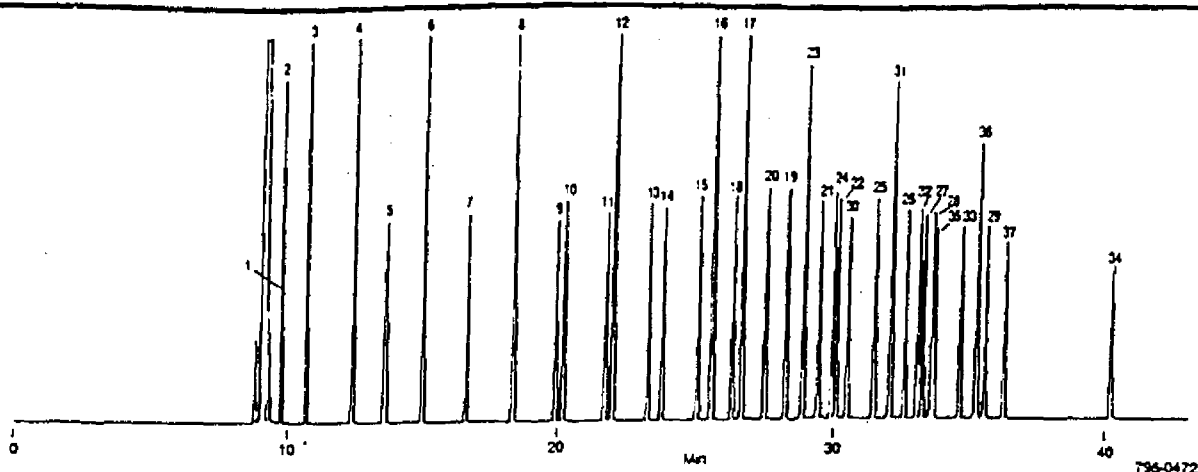
14-4 figure Gas chromatogram of separation of 37 fatty acid methyl esters (FAMEs) on a SP-2650 column. Peaks are identified in Table 14-3. (Reprinted with permission of Supelco, Inc., Bellefonte, PA. Figure 795-0472 from Bulletin 907.)

14-3 table Composition of Supelco 37 Component Fatty Acid Methyl Ester (FAME) Mix[1]

Peak ID	Component (Acid Methyl Esters)	Peak ID	Component (Acid Methyl Esters)
1	C4:0 (Butyric)	20	C18:2n6t (Linolelaidic)
2	C6:0 (Caproic)	21	C18:3n6 (γ-Linolenic)
3	C8:0 (Caprylic)	22	C18:3n3 (α-Linolenic)
4	C10:0 (Capric)	23	C20:0 (Arachidic)
5	C11:0 (Undecanoic)	24	C20:1n9 (cis-11-Eicosenoic)
6	C12:0 (Lauric)	25	C20:2 (cis-11, 14-Eicosadienoic)
7	C13:0 (Tridecanoic)	26	C20:3n6 (cis-8, 11, 14-Eicosatrienoic)
8	C14:0 (Myristic)	27	C20:3n3 (cis-11, 14, 17-Eicosatrienoic)
9	C14:1 (Myristoleic)	28	C20:4n6 (Arachidonic)
10	C15:0 (Pentadecanoic)	29	C20:5n3 (cis-5, 8, 11, 14, 17-Eicosapentaenoic)
11	C15:1 (cis-10-Pentadecenoic)	30	C21:0 (Henicosanoic)
12	C16:0 (Palmitic)	31	C22:0 (Behenic)
13	C16:1 (Palmitoleic)	32	C22:1n9 (Behenic)
14	C17:0 (Heptadecanoic)	33	C22:2 (cis-13, 16-Docosadienoic)
15	C17:1 (cis-10-Heptadecenoic)	34	C22:6n3 (cis-4, 7, 10, 13, 16, 19-Docosa-
16	C18:0 (Stearic)		hexaenoic)
17	C18:1n9c (Oleic)	35	C23:0 (Tricosanoic)
18	C18:1n9t (Elaidic)	36	C24:0 (Lignoceric)
19	C18:2n6c (Linoleic)	37	C24:1n9 (Nervonic)

[1]Chromatogram for peaks is Fig. 14-4.
Reprinted with permission of Supelco, Inc., Bellafonte. PA. Bulletin 907.

acid.) An aliquot of the upper heptane solution is removed and dried with anhydrous Na_2SO_4, then diluted to a concentration of 5–10% for injection onto the GC.

14.6.1.3 Applications

Determination of the fatty acid composition of a product permits the calculation of the following categories

of fats that pertain to health issues and food labeling: percent saturated fatty acids, percent unsaturated fatty acids, percent monounsaturated fatty acids, percent polyunsaturated fatty acids, and percent *trans* isomer fatty acids. However, some of these categories are more accurately determined using other methods. For example, separation of all *trans* and *cis* isomers (as well as geometric isomers) is difficult using GC analysis alone since many combinations of isomers and fatty acids are

possibly present in a lipid sample (13). Similar to fatty acids, the mono-, di-, and triacylglycerols may be determined using capillary GC. Short columns and high temperatures are needed for analysis of intact triacylglycerols.

14.6.2 cis, cis-Polyunsaturated Fatty Acids (PUFAs)

Although a determination of fatty acid composition provides a means of calculating the amount of polyunsaturated fatty acids present in a sample, it is not specific for the cis, cis-polyunsaturated fatty acids, which nutrition labeling requires.

14.6.2.1 Principle

Saponified fatty acids are treated with lipoxidase. The enzyme is specific for the cis, cis 1,4-methylene interrupted diene structure (—CH=CH—CH₂—CH=CH—), which is converted to a conjugated diene (—CH=CH—CH=CH—CH₂—) and measured spectrophotometrically.

14.6.2.2 Procedure

The lipid extract is saponified and reacted with lipoxidase. After 30 min the absorbance of the solution is read at 234 nm.

14.6.2.3 Applications

PUFAs are legally defined as fatty acids having cis, cis methylene-interrupted double bonds. Though it requires time and effort, this enzymatic method is necessary for the proper determination of PUFAs for nutrition labeling purposes.

14.6.3 trans Isomer Fatty Acids

Most natural fats and oils extracted from plant sources contain only isolated (i.e., nonconjugated), cis double bonds. Fats and oils extracted from animal sources may contain small amounts of trans double bonds. Inasmuch as the trans isomer is more thermodynamically stable, additional amounts of trans double bonds can be formed in fats and oils that undergo oxidation, or during processing treatments such as extraction, heating, and hydrogenation. Ongoing studies are evaluating the health effects of dietary lipids that contain trans fatty acids.

14.6.3.1 Principle

The concentration of trans fatty acids is measurable in lipids from an absorption peak at 966 cm⁻¹ in the infrared spectrum.

14.6.3.2 Procedure

AOCS Method Cd 14-95 requires liquid samples be converted to methyl esters and dissolved in an appropriate solvent, for example, carbon disulfide. The absorbance spectra between 1050 and 900 cm⁻¹ is obtained using an infrared spectrometer (see Chapter 27). Methyl elaidate is used as an external standard in calculating the content of trans double bonds. Alternately, AOCS Recommended Practice Cd 14d-96 determines total trans fatty acids using attenuated total reflection–Fourier transform infrared (ATR–FTIR) spectroscopy (see Chapter 27).

14.6.3.3 Applications

The method described will detect only isolated (i.e., nonconjugated) trans isomers. This is especially important when oxidized samples are of interest since oxidation results in a conversion from nonconjugated to conjugated double bonds. Also, the method is restricted to samples containing at least 0.5% trans isomers. For samples containing less than 0.5% trans double bonds, a capillary GC method (AOCS method Ce 1c-89) is recommended.

14.6.4 Cholesterol

Many methods exist for the quantification of cholesterol in various matrices. Consulting research literature will give an indication of current practice and methods that may be less laborious or adapted for use with specific foodstuffs.

14.6.4.1 Principle

The lipid extracted from the food is saponified. Cholesterol (in the nonsaponifiable fraction) is extracted and derivatized to form trimethylsilyl (TMS) ethers. Quantitation is achieved using capillary GC.

14.6.4.2 Procedure

AOAC Method 976.26 outlined here is representative of the various procedures available for cholesterol determination. Lipids are extracted from the food, then saponified, and the unsaponifiable fraction is extracted. This is accomplished by filtering an aliquot of the chloroform layer through anhydrous sodium sulfate and evaporating to dryness in a water bath using a stream of nitrogen gas. Concentrated potassium hydroxide and ethanol are added and the solution is refluxed. Aliquots of benzene and 1 N potassium hydroxide are added, then shaken. The aqueous layer is removed and the process is repeated with 0.5 N potassium hydroxide. After several washes with water,

the benzene layer is dried with anhydrous sodium sulfate and an aliquot is evaporated to dryness on a rotary evaporator. The residue is taken up in dimethylformamide. An aliquot of this sample is derivatized by adding hexamethyldisilazane and trimethylchlorosilane. Water and an internal standard in heptane are added, then the solution is centrifuged. A portion of the heptane layer is injected into a GC.

14.6.4.3 Applications

GC quantitation of cholesterol is recommended since many spectrophometric methods are not specific for cholesterol. Other GC, HPLC, and enzymatic methods are available. For example, cholesterol methods developed for frozen foods (26) and meat products (27) eliminate the fat extraction step, directly saponifying the sample; compared to the AOAC method outlined previously, they are more rapid and avoid exposure to toxic solvents.

Cholesterol oxidation products can be measured using GC, HPLC, and TLC.

14.6.5 Separation of Lipid Fractions by TLC

14.6.5.1 Procedure

TLC is performed using silica gel G as the adsorbent and hexane–diethyl ether–formic acid (80:20:2, by vol) as the eluting solvent system (Fig 14-5). Plates are sprayed with 2',7'-dichlorofluorescein in methanol and placed under ultraviolet light to view yellow bands against a dark background (5).
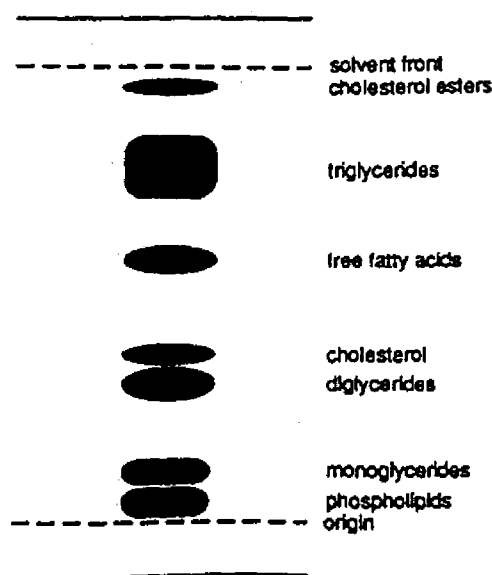
14-5 figure Schematic thin-layer chromatography (TLC) separation of lipid fractions on Silica Gel G. [Adapted with permission from (5).]

14.6.5.2 Applications

This procedure permits rapid analysis of the presence of the various lipid fractions in a food lipid extract. For small-scale preparative purposes, TLC plates can be scraped to remove various bands for further analysis using GC or other means. Many variations in TLC parameters are available that will separate various lipids.

14.7 SUMMARY

The importance of fat characterization is evident in many aspects of the food industry, including ingredient technology, product development, quality assurance, product shelf life, and regulatory aspects. The effort to reduce the amount of calories consumed as fat in the United States accentuates the significance of understanding the lipid components of food.

The methods described in this chapter help to characterize bulk oils and fats and the lipids in foodstuffs. Methods described for bulk oils and fats can be used to determine characteristics such as melting point; smoke, flash, and fire points; color; degree of unsaturation; average fatty acid chain length; and amount of polar components. The peroxide value, TBA, and hexanal tests can be used to measure the present status of a lipid with regard to oxidation, while the OSI can be used to predict the susceptibility of a lipid to oxidation and the effectiveness of antioxidants. Lipid fractions, including fatty acids, triacylglycerols, phospholipids, and cholesterol, are commonly analyzed by chromatographic techniques such as GC and TLC.

The methods discussed in this chapter represent only a few of the many tests that have been developed to characterize lipid material. Consult the references cited for additional methods or more detailed explanations. Time, funding, availability of equipment and instruments, required accuracy, and purpose all will dictate the choice of method to characterize oils, fats, and foodstuffs containing lipids.

14.8 STUDY QUESTIONS

1. You want to compare several fat/oil samples for the chemical characteristics listed below. For each characteristic, name one test (give full name, not abbreviation) that could be used to obtain the information desired:
   a. degree of unsaturation
   b. predicted susceptibility to oxidative rancidity
   c. present status with regard to oxidative rancidity
   d. average fatty acid molecular weight
   e. amount of solid fat at various temperatures
   f. hydrolytic rancidity
2. Your analysis of an oil sample gives the following results.

What do each of these results tell you about the characteristics of the sample? Briefly describe the principle for each method used.

a. large saponification value
b. low iodine value
c. high TBA number
d. high free fatty acid content
e. high oil stability index

3. Define solid fat content and explain the usefulness of this measurement.

4. Peroxide value, TBA number, and hexanal content all can be used to help characterize a fat sample.
a. What do the results of these tests tell you about a fat sample?
b. Differentiate these three tests as to what chemical is being measured.

5. What methods would be useful in determining the effectiveness of various antioxidants added to an oil?

6. You are responsible for writing the specifications for vegetable oil purchased from your supplier for use in deep-fat frying several foods processed by your company. Itemize the tests you should require in your list of specifications (specific values for the tests are not needed). For each test, briefly state what useful information is obtained.

7. The Nutrition Education and Labeling Act of 1990 (see Chapter 3) requires that the nutrition label on food products contains information related to lipid constituents. In addition to the amount of *total fat* (see Chapter 13), the label must state the amount of *saturated fat* and the *cholesterol* content.
a. For a product such as traditional potato chips, explain an appropriate method for the analysis of each of these lipid constituents.
b. Compared to assays on traditional chips, how would the assays for total fat and saturated fat differ for potato chips made with Olestra⁵?

14.9 PRACTICE PROBLEMS

1. A 5.00-g sample of oil was saponified with excess KOH. The unreacted KOH was then titrated with 0.500 N HCl (standardized). The difference between the blank and the sample was 25.8 ml of titrant. Calculate the saponification value.

2. A sample (5.0 g) of food grade oil was reacted with excess KI to determine peroxide value. The free iodine was titrated with a standardized solution of 0.10 N Na$_2$S$_2$O$_3$. The amount of titrant required was 0.60 ml (blank corrected). Calculate the peroxide value of the oil.

Answers

1. 145; 2. 12.

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Protein Analysis

Sam K. C. Chang

15.1 INTRODUCTION

15.1.1 Classification and General Considerations

Proteins are an abundant component in all cells, and almost all except storage proteins are important for biological functions and cell structure. Food proteins are very complex. Many have been purified and characterized. Proteins vary in molecular mass, ranging from approximately 5000 to more than a million daltons. They are composed of elements including hydrogen, carbon, nitrogen, oxygen, and sulfur. Twenty α-amino acids are the building blocks of proteins; the amino acid residues in a protein are linked by peptide bonds. Nitrogen is the most distinguishing element present in proteins. However, nitrogen content in various food proteins ranges from 13.4% to 19.1% (1) due to the variation in the specific amino acid composition of proteins. Generally, proteins rich in basic amino acids contain more nitrogen.

Proteins can be classified by their composition, structure, biological function, or solubility properties. For example, simple proteins contain only amino acids upon hydrolysis, but conjugated proteins also contain non-amino-acid components.

Proteins have unique conformations that could be altered by denaturants such as heat, acid, alkali, 8 M urea, 6 M guanidine-HCl, organic solvents, and detergents. The solubility as well as functional properties of proteins could be altered by denaturants.

The analysis of proteins is complicated by the fact that some food components possess similar physico-chemical properties. Nonprotein nitrogen could come from free amino acids, small peptides, nucleic acids, phospholipids, amino sugars, porphyrin, and some vitamins, alkaloids, uric acid, urea, and ammonium ions. Therefore, the total organic nitrogen in foods would represent nitrogen primarily from proteins and to a lesser extent from all organic nitrogen-containing nonprotein substances. Depending upon methodology, other major food components, including lipids and carbohydrates, may interfere physically with analysis of food proteins.

Numerous methods have been developed to measure protein content. The basic principles of these methods include the determinations of nitrogen, peptide bonds, aromatic amino acids, ultraviolet absorptivity of proteins, free amino groups, light scattering properties, and dye-binding capacity. In addition to factors such as sensitivity, accuracy, precision, speed, and cost of analysis, what is actually being measured must be considered in the selection of an appropriate method for a particular application.

15.1.2 Importance of Analysis

Protein analysis is important for:

1. Biological activity determination. Some proteins, including enzymes or enzyme inhibitors, are relevant to food science and nutrition: for instance, the proteolytic enzymes in the tenderization of meats, pectinases in the ripening of fruits, and trypsin inhibitors in legume seeds are proteins.
2. Functional property investigation. Proteins in various types of food have unique food functional properties: for example, gliadin and glutenins in wheat flour for breadmaking, casein in milk for coagulation into cheese products, and egg albumen for foaming.
3. Nutrition labeling.

Protein analysis is required when you want to know:

1. Total protein content
2. Amino acid composition
3. Content of a particular protein in a mixture
4. Protein content during isolation and purification of a protein
5. Nonprotein nitrogen
6. Nutritive value (digestibility, protein efficiency ratio, or nitrogen balance) of a protein

15.1.3 Content in Foods

Protein content in food varies widely. Foods of animal origin and legumes are excellent sources of proteins. The protein contents of selected food items are listed in Table 15-1.

15.2 METHODS

Principles, general procedures, and applications are described below for various protein determination methods. Refer to the referenced methods for detailed instructions of the procedures. Several of the methods cited are from the *Official Methods of Analysis* of AOAC International (3). Many of the methods covered are described in somewhat more detail in a recent book on food proteins (4).

15.2.1 Kjeldahl Method

15.2.1.1 Principle

In the Kjeldahl procedure, proteins and other organic food components in a sample are digested with sulfuric acid in the presence of catalysts. The total organic

15-1 table Protein Content of Selected Foods

Food Item	Percent Protein (wet weight basis)
Cereals and pasta	
Rice, brown, long-grain raw	7.9
Rice, white, long-grain, regular, raw, enriched	7.1
Wheat flour, whole-grain	13.7
Corn flour, whole-grain, yellow	6.9
Spaghetti, dry, enriched	12.8
Cornstarch	0.3
Dairy products	
Milk, whole, fluid	3.3
Milk, skim, dry	36.2
Cheese, cheddar	24.9
Yogurt, plain, low fat	5.3
Fruits and vegetables	
Apple, raw, with skin	0.2
Asparagus, raw	2.3
Strawberries, raw	0.6
Lettuce, iceberg, raw	1.0
Potato, whole, flesh and skin	2.1
Legumes	
Soybeans, mature seeds, raw	36.5
Beans, kidney, all types, mature seeds, raw	23.6
Tofu, raw, firm	15.8
Tofu, raw, regular	8.1
Meats, poultry, fish	
Beef, chuck, arm pot roast	19.5
Beef, cured, dried beef	29.1
Chicken, broilers or fryers, breast meat only, raw	23.1
Ham, sliced, regular	17.6
Egg, raw, whole	12.5
Finfish, cod, Pacific, raw	17.9
Finfish, tuna, white, canned in oil, drained solids	26.5

From USDA Nutrient Database for Standard Reference. Release 11-1 (August 1997) http://www.nal.usda.gov/fnic/cgi-bin/nut_search.pl

nitrogen is converted to ammonium sulfate. The digest is neutralized with alkali and distilled into a boric acid solution. The borate anions formed are titrated with standardized acid, which is converted to nitrogen in the sample. The result of the analysis represents the crude protein content of the food since nitrogen also comes from nonprotein components.

15.2.1.2 Historical Background

15.2.1.2.1 Original Method In 1883, Johann Kjeldahl developed the basic process of today's Kjeldahl method to analyze organic nitrogen. General steps in the original method include:

1. Digestion with sulfuric acid, with the addition

of powdered potassium permanganate to complete oxidation and conversion of nitrogen to ammonium sulfate.
2. Neutralization of the diluted digest, followed by distillation into a known volume of standard acid, which contains potassium iodide and iodate.
3. Titration of the liberated iodine with standard sodium thiosulfate.

15.2.1.2.2 Improvements Several important modifications have improved the original Kjeldahl process:

1. Metallic catalysts such as mercury, copper, and selenium are added to sulfuric acid for complete digestion. Mercury has been found to be the most satisfactory. Selenium dioxide and copper sulfate in the ratio of 3:1 have been reported to be effective for digestion. Copper and titanium dioxide also have been used as a mixed catalyst for digestion (AOAC Method 988.05) (3). The use of titanium dioxide and copper poses less safety concern than mercury in the post analysis disposal of the waste.
2. Potassium sulfate is used to increase the boiling point of the sulfuric acid to accelerate digestion.
3. Sulfide or sodium thiosulfate are added to the diluted digest to help release nitrogen from mercury, which tends to bind ammonium.
4. The ammonia is distilled directly into a boric acid solution, followed by titration with standard acid.
5. Colorimetry, Nesslerization, or ion chromatography to measure ammonia is used to determine nitrogen content after digestion.

An excellent book to review the Kjeldahl method for total organic nitrogen was written by Bradstreet (5). The basic AOAC Kjeldahl procedure is Method 955.04. Semiautomation, automation, and modification for microgram nitrogen determination (micro Kjeldahl method) have been established by AOAC in Methods 976.06, 976.05 and 960.52, respectively.

15.2.1.3 General Procedures and Reactions

15.2.1.3.1 Sample Preparation Solid foods are ground to pass a 20 mesh screen. Samples for analysis should be homogeneous. No other special preparations are required.

15.2.1.3.2 Digestion Place sample (accurately weighed) in a Kjeldahl flask. Add acid and catalyst; digest until clear to get complete breakdown of all

organic matter. Nonvolatile ammonium sulfate is formed from the reaction of nitrogen and sulfuric acid.

$$\text{Protein} \xrightarrow[\text{Heat, catalyst}]{\text{Sulfuric acid}} (NH_4)_2SO_4 \qquad [1]$$
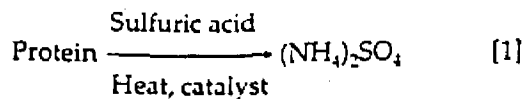
During digestion, protein nitrogen is liberated to form ammonium ions; sulfuric acid oxidizes organic matter and combines with ammonium formed; carbon and hydrogen elements are converted to carbon dioxide and water.

15.2.1.3.3 Neutralization and Distillation
The digest is diluted with water. Alkali-containing sodium thiosulfate is added to neutralize the sulfuric acid. The ammonia formed is distilled into a boric acid solution containing the indicators methylene blue and methyl red.

$$(NH_4)_2SO_4 + 2NaOH \rightarrow 2NH_3 + Na_2SO_4 + 2H_2O \qquad [2]$$

$$\underset{\text{(boric acid)}}{NH_3 + H_3BO_3} \rightarrow \underset{\text{(borate ion)}}{NH_4 + H_2BO^-_3} \qquad [3]$$
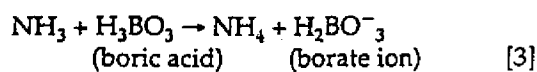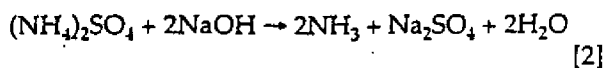
15.2.1.3.4 Titration
Borate anion (proportional to the amount of nitrogen) is titrated with standardized HCl.

$$H_2BO^-_3 + H^+ \rightarrow H_3BO_3 \qquad [4]$$
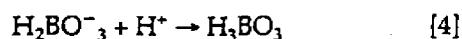
15.2.1.3.5 Calculations

$$\begin{aligned}\text{Moles of HCl} &= \text{moles NH}_3 \\ &= \text{moles N in the sample}\end{aligned} \qquad [5]$$

A reagent blank should be run to subtract reagent nitrogen from the sample nitrogen.

$$\begin{aligned}\% N = N \text{ HCl} &\times \frac{\text{Corrected acid volume}}{\text{g of sample}} \\ &\times \frac{14\,\text{g N}}{\text{mole}} \times 100\end{aligned} \qquad [6]$$

where:

N HCl = normality of HCl, in moles/1000 ml

Corrected acid vol. = (ml std. acid for sample) – (ml std. acid for blank)

14 = atomic weight of nitrogen

A factor is used to convert percent N to percent crude protein. Most proteins contain 16% N, so the conversion factor is 6.25 (100/16 = 6.25).

$$\begin{aligned}\%N/0.16 &= \% \text{ protein} \\ &\text{or} \\ \% N \times 6.25 &= \% \text{ protein}\end{aligned} \qquad [7]$$

Conversion factors for various foods are given in Table 15-2.

15-2
table
Nitrogen to Protein Conversion Factors for Various Foods

	Percent N in Protein	Factor
Egg or meat	16.0	6.25
Milk	15.7	6.38
Wheat	18.76	5.33
Corn	17.70	5.65
Oat	18.66	5.36
Soybean	18.12	5.52
Rice	19.34	5.17

Data from (1,6).

15.2.1.3.6 Alternate Procedures
In place of distillation and titration with acid, ammonia or nitrogen can be quantitated by:

1. Nesslerization

$$\underset{\substack{\text{mercuric}\\\text{iodide}}}{4NH_4OH + 2HgI_2} + 4KI + 3KOH \rightarrow \underset{\substack{\text{ammonium dimercuric}\\\text{iodide, red-orange, 440 nm}}}{NH_2Hg_2IO} + 7KI + 2H_2O \qquad [8]$$
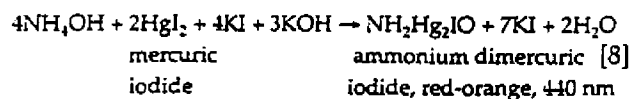
This method is rapid and sensitive, but the ammonium dimercuric iodide is colloidal and color is not stable.

2. $\underset{OH^-}{NH_3 + \text{phenol} + \text{hypochlorite}} \rightarrow \text{indophenol (blue, 630 nm)} \qquad [9]$

3. pH measurement after distillation into known volume of boric acid.

4. Direct measurement of ammonia, using ion chromatographic method

15.2.1.4 Applications

Advantages:

1. Applicable to all types of foods
2. Relatively simple
3. Inexpensive
4. Accurate; an official method for crude protein content
5. Has been modified (micro Kjeldahl method) to measure microgram quantities of proteins.

Disadvantages:

1. Measures total organic nitrogen, not just protein nitrogen.
2. Time consuming (at least 2 hr to complete)
3. Poorer precision than the biuret method
4. Corrosive reagent

15.2.2 Biuret Method

15.2.2.1 Principle

A violet-purplish color is produced when cupric ions are complexed with peptide bonds (substances containing at least two peptide bonds, i.e., biuret, large peptides, and all proteins) under alkaline conditions. The absorbance of the color produced is read at 540 nm. The color intensity (absorbance) is proportional to the protein content of the sample (7).

15.2.2.2 Procedure

1. A 5-ml biuret reagent is mixed with a 1-ml portion of protein solution (1–10 mg protein/ml). The reagent includes copper sulfate, NaOH, and potassium sodium tartrate, which is used to stabilize the cupric ion in the alkaline solution.
2. After the reaction mixture is allowed to stand at room temperature for 15 or 30 min, the absorbance is read at 540 nm against a reagent blank.
3. Filtration or centrifugation before reading absorbance is required if the reaction mixture is not clear.
4. A standard curve of concentration versus absorbance is constructed using bovine serum albumin (BSA).

15.2.2.3 Applications

The biuret method has been used to determine proteins in cereal (8, 9), meat (10), soybean proteins (10), and as a qualitative test for animal feed [AOAC Method 935.11 (refers to Methods 22.012–22.013, AOC, 10th ed., 1965)] (12). The biuret method also can be used to measure the protein content of isolated proteins.

Advantages:

1. Less expensive than the Kjeldahl method; rapid (can be completed in less than 30 min); simplest method for analysis of proteins.
2. Color deviations are encountered less frequently than with Lowry, ultraviolet (UV) absorption, or turbidimetric methods (described below).
3. Very few substances other than proteins in foods interfere with the biuret reaction.
4. Does not detect nitrogen from nonpeptide or nonprotein sources.

Disadvantages:

1. Not very sensitive as compared to the Lowry method; requires at least 2–4 mg protein for assay.

2. Absorbance could be contributed from bile pigments if present.
3. High concentration of ammonium salts interfere with the reaction.
4. Color varies with different proteins; gelatin gives a pinkish-purple color.
5. Opalescence could occur in the final solution if high levels of lipid or carbohydrate are present.
6. Not an absolute method: color must be standardized against known protein (e.g., BSA) or against the Kjeldahl nitrogen method.

15.2.3 Lowry Method

15.2.3.1 Principle

The Lowry method (13,14) combines the biuret reaction with the reduction of the Folin–Ciocalteau phenol reagent (phosphomolybdic–phosphotungstic acid) by tyrosine and tryptophan residues in the proteins. The bluish color developed is read at 750 nm (high sensitivity for low protein concentration) or 500 nm (low sensitivity for high protein concentration). The original procedure has been modified by Miller (15) and Hartree (16) to improve the linearity of the color response to protein concentration.

15.2.3.2 Procedure

The following procedure is based on the modified procedure of Hartree (16):

1. Proteins to be analyzed are diluted to an appropriate range (20–100 μg).
2. K Na Tartrate–Na_2CO_3 solution is added after cooling and incubated at room temperature for 10 min.
3. $CuSO_4$–K Na Tartrate–NaOH solution is added after cooling and incubated at room temperature for 10 min.
4. Freshly prepared Folin reagent is added, then the reaction mixture is mixed and incubated at 50°C for 10 min.
5. Absorbance is read at 650 nm.
6. A standard curve of BSA is carefully constructed for estimating protein concentration of the unknown.

15.2.3.3 Applications

Because of its simplicity and sensitivity, the Lowry method has been widely used in protein biochemistry. However, it has not been widely used to determine proteins in food systems without first extracting the proteins from the food mixture.

Advantages:

1. Very sensitive
   a. 50–100 times more sensitive than biuret method
   b. 10–20 times more sensitive than 280 nm UV absorption method (described below)
   c. Several times more sensitive than ninhydrin method (described below)
   d. Similar sensitivity as Nesslerization; however, more convenient than Nesslerization
2. Less affected by turbidity of the sample
3. More specific than most other methods
4. Relatively simple; can be done in 1–1.5 hr.

Disadvantages:
For the following reasons, the Lowry procedure requires careful standardization for particular applications:

1. Color varies with different proteins to a greater extent than the biuret method.
2. Color is not strictly proportional to protein concentration.
3. The reaction is interfered with to varying degrees by sucrose, lipids, phosphate buffers, monosaccharides, and hexoamines.
4. High concentrations of reducing sugars, ammonium sulfate, and sulfhydryl compounds interfere with the reaction.

15.2.4 Bicinchoninic Acid (BCA) Method

15.2.4.1 Principle

Smith et al. (17) proposed that proteins reduce cupric ions to cuprous ions under alkaline conditions. The cuprous ion complexes with apple-greenish BCA reagent to form a purplish color. The color formed is proportional to protein concentration.

15.2.4.2 Procedure

1. Mix (one step) the protein solution with the BCA reagent, which contains BCA sodium salt, sodium carbonate, NaOH, and copper sulfate, pH 11.25.
2. Incubate at 37°C for 30 min, or room temperature for 2 hr, or 60°C for 30 min. The selection of the temperature depends upon sensitivity desired. A higher temperature gives a greater color response.
3. Read the solution at 562 nm against a reagent blank.
4. Construct a standard curve using BSA.

15.2.4.3 Applications

The BCA method has been used in protein isolation and purification. The suitability of this procedure for measuring protein in complex food systems has not been reported.

Advantages:

1. Sensitivity is comparable to that of the Lowry method; sensitivity of the micro BCA method (0.5–10 µg) is slightly better than that of the Lowry method.
2. One-step mixing is easier than in the Lowry method.
3. The reagent is more stable than for the Lowry reagent.
4. Nonionic detergent and buffer salts do not interfere with the reaction.
5. Medium concentrations of denaturing reagents (4 M guanidine-HCl or 3 M urea) do not interfere.

Disadvantages:

1. Color is not stable with time. The analyst needs to carefully control the time for reading absorbance.
2. Reducing sugars interfere to a greater extent than in the Lowry method. High concentrations of ammonium sulfate also interfere.
3. Color variations among proteins are similar to those in the Lowry method.
4. Response of absorbance to concentration is not linear.

15.2.5 Ultraviolet (UV) 280 nm Absorption Method

15.2.5.1 Principle

Proteins show strong absorption at UV 280 nm, primarily due to tryptophan and tyrosine residues in the proteins. Because the content of tryptophan and tyrosine in proteins from each food source is fairly constant, the absorbance at 280 nm could be used to estimate the concentration of proteins, using Beer's law. Since each protein has a unique aromatic amino acid composition, the extinction coefficient (E_{280}) or molar absorptivity (E_m) must be determined for individual proteins for protein content estimation.

15.2.5.2 Procedure

1. Proteins are solubilized in buffer or alkali.
2. Absorbance of protein solution is read at 280 nm against a reagent blank.

3. Protein concentration is calculated according to the equation

$$A = abc \qquad [10]$$

where:

A = absorbance
a = absorptivity
b = cell or cuvette path length
c = concentration

15.2.5.3 Applications

The UV 280 nm method has been used to determine the protein contents of milk (18) and meat products (19). It has not been used widely in food systems. This technique is better applied in a purified protein system or to proteins that have been extracted in alkali or denaturing agents such as 8 M urea. Although peptide bonds in proteins absorb more strongly at 190–220 nm than at 280 nm, the low UV region is more difficult to measure.

Advantages:

1. Rapid and relatively sensitive (At 280 nm, 100 µg or more protein are required; several times more sensitive than the biuret method.)
2. No interference from ammonium sulfate and other buffer salts
3. Nondestructive; samples can be used for other analyses after protein determination; used very widely in post-column detection of proteins.

Disadvantages:

1. Nucleic acids also absorb at 280 nm. The absorption 280 nm/260 nm ratios for pure protein and nucleic acids are 1.75 and 0.5, respectively. One can correct the absorption of nucleic acids at 280 nm if the ratio of the absorption of 280 nm/260 nm is known. Nucleic acids also can be corrected using a method based on the absorption difference between 235 nm and 280 nm (20).
2. Aromatic amino acid contents in the proteins from various food sources differ considerably.
3. The solution must be clear and colorless. Turbidity due to particulates in the solution will increase absorbance falsely.
4. A relatively pure system is required to use this method.

15.2.6 Dye Binding Method

15.2.6.1 Anionic Dye Binding

15.2.6.1.1 Principle The protein-containing sample is mixed with a known excess amount of anionic dye in a buffered solution. Proteins bind the dye to form an

insoluble complex. The unbound soluble dye is measured after equilibration of the reaction and the removal of insoluble complex by centrifugation or filtration.

protein + excess dye → protein–dye insoluble
complex + [11]
unbound soluble dye
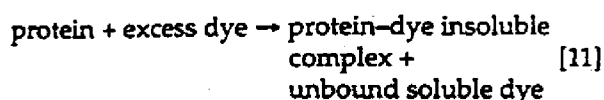
The anionic sulfonic acid dye, including acid orange 12, orange G, and Amido black 10B, binds cationic groups of the basic amino acid residues (imidazole of histidine, guanidine of arginine, and ε-amino group of lysine) and the free amino terminal group of the protein. The amount of unbound dye is inversely related to the protein content of the sample (21).

15.2.6.1.2 Procedure

1. The sample is finely ground (60 mesh or smaller sizes) and added to an excess dye solution.
2. The content is shaken vigorously to equilibrate the dye binding reactions and filtered or centrifuged to remove insoluble substances.
3. Absorbance of the unbound dye solution in the filtrate is measured and dye concentration estimated from a dye standard curve.
4. A straight calibration curve can be obtained by plotting the unbound dye concentration against total nitrogen (as determined by Kjeldahl method) of a given food covering a wide range of protein content.
5. Protein content of the unknown sample of the same food type can be estimated from the calibration curve or from a regression equation calculated by the least squares method.

15.2.6.1.3 Applications Anionic dye binding has been used to estimate proteins in milk (22,23), wheat flour (24), soy products (11), and meats (10). The AOAC includes two dye-binding methods (Method 967.12 using Acid Orange 12 and Method 975.17 using Amido Black 10B) for analyzing proteins in milk.

Advantages:

1. Rapid (15 min or less), inexpensive, and relatively accurate for analyzing protein content in food commodities
2. May be used to estimate the changes in available lysine content of cereal products during processing since the dye does not bind altered, unavailable lysine. Since lysine is the limiting amino acid in cereal products, the available lysine content represents protein nutritive value of the cereal products (25).
3. No corrosive reagents
4. Does not measure nonprotein nitrogen.
5. More precise than the Kjeldahl method

Disadvantages:

1. Not sensitive; milligram quantities of protein are required.
2. Proteins differ in basic amino acid content and so differ in dye-binding capacity. Therefore, a calibration curve for a given food commodity is required.
3. Some nonprotein components bind dye (i.e., starch) or protein (i.e., calcium or phosphate) and cause errors in final results. The problem with calcium and heavy metal ions can be eliminated using properly buffered reagent that contains oxalic acid.

15.2.6.2 Bradford Method

15.2.6.2.1 Principle When Coomassie Brilliant Blue G-250 binds to protein, the dye changes color from reddish to bluish, and the absorption maximum of the dye is shifted from 465 to 595 nm. The change in the absorbance at 595 nm is proportional to the protein concentration of the sample (26).

15.2.6.2.2 Procedure

1. Coomassie Brilliant Blue G-250 is dissolved in 95% ethanol and acidified with 85% phosphoric acid.
2. Samples containing proteins (1–100 µg/ml) and standard BSA solutions are mixed with the Bradford reagent.
3. Absorbance at 595 nm is read against a reagent blank.
4. Protein concentration in the sample is estimated from the BSA standard curve.

15.2.6.2.3 Applications The Bradford method has been used successfully to determine protein content in worts and beer products (27) and in potato tubers (28). This procedure has been improved to measure microgram quantities of proteins (29). Due to its rapidity, sensitivity, and fewer interferences than the Lowry method, the Bradford method has been used widely in protein purification.

Advantages:

1. Rapid; reaction can be completed in 2 min.
2. Reproducible
3. Sensitive; several-fold more sensitive than the Lowry method
4. No interference from cations such as K^+, Na^+, and Mg^{2+}
5. No interference from ammonium sulfate
6. No interference from polyphenols and carbohydrate such as sucrose
7. Measures protein or peptides with molecular

mass approximately equal to or greater than 4000 daltons.

Disadvantages:

1. Interfered with by both nonionic and ionic detergents, such as Triton X-100 and sodium dodecyl sulfate. However, errors due to small amounts (0.1%) of these detergents can be corrected using proper controls.
2. The protein–dye complex can bind to quartz cuvettes. The analyst must use glass or plastic cuvettes.
3. Color varies with different types of proteins. The standard protein must be selected carefully.

15.2.7 Ninhydrin Method

15.2.7.1 Principle

Amino acids, ammonia, and primary amino groups in a protein, when boiled in a pH 5.5 buffer in the presence of ninhydrin and hydrindantin, form a Ruhemann purple color (30,31).

15.2.7.2 Procedure

1. Mix a 1-ml sample solution with 1 ml of ninhydrin solution in a test tube.
2. Heat in a boiling bath for 15 min.
3. Add 5 ml of ethanol or propanol diluent, shake, and cool.
4. Read absorbance at 570 nm against a water blank.

15.2.7.3 Applications

The ninhydrin method has not been used widely for the determination of protein quantity in foods. However, it can be used to determine the hydrolysis of peptide bonds during food processing and to quantitate amino acids.

Advantage:

1. Relatively rapid as compared to the Kjeldahl method

Disadvantages:

1. The presence of a small quantity of amino acids, peptides, primary amines, and ammonia causes an overestimation of the protein content.
2. Low precision
3. Color varies with different amino acid compositions. Proline absorbs maximum at 440 nm; other amino acids at 570 nm.
4. A standard calibration curve must be prepared on each occasion.

15.2.8 Turbidimetric Method

15.2.8.1 Principle

Low concentrations (3–10%) of trichloroacetic acid, sulfosalicylic acid (32,33), and potassium ferricyanide in acetic acid (34) can be used to precipitate extracted proteins to form a turbid suspension of protein particles. The turbidity can be measured from the reduction in the transmission of radiation. The reduction in radiation transmission is due to radiation scattering by the protein particles. The intensity of the radiation reduction can be related to protein concentration in the solution.

15.2.8.2 Procedure

The general procedure for measuring wheat proteins by the sulfosalicylic acid method (33) is as follows:

1. Wheat flour is extracted with 0.05 N sodium hydroxide.
2. Protein solubilized in alkali is separated from the nonsoluble materials by centrifugation.
3. Sulfosalicylic acid is mixed with a portion of the protein solution.
4. The degree of turbidity is measured by reading the light transmittance at 540 nm against a reagent blank.
5. The protein content can be estimated from a calibration curve, which is established using the Kjeldahl nitrogen method.

15.2.8.3 Applications

The turbidimetric method has been used to measure protein content of wheat flour (33) and corn (35).
Advantages:

1. Rapid; can be completed in 15 min.
2. Does not measure nonprotein nitrogen other than that in nucleic acids.

Disadvantages:

1. Different proteins precipitate at different rates.
2. Turbidity varies with different concentrations of acid reagents.
3. Nucleic acids also are precipitated by acid reagents.

15.2.9 Dumas (Combustion) Method

15.2.9.1 Principle

Samples are combusted at high temperatures (700–800°C). The nitrogen released is quantitated by gas chromatography using a thermal conductivity detector (TCD). The nitrogen determined is converted to protein content in the sample.

15.2.9.2 Procedure

Samples (approximately 100–500 mg) are weighed into a tin capsule and introduced to a combustion reactor in an automated equipment. The nitrogen released is measured by a built-in gas chromatograph.

15.2.9.3 Applications

The combustion method is suitable for all types of foods. AOAC Method 992.15 and Method 992.23 are for meat and cereal grains, respectively.
Advantages:

1. The combustion method is an alternative to the Kjeldahl method.
2. Requires no hazardous chemicals.
3. Can be accomplished in 3 min.
4. Recent automated instruments can analyze up to 150 samples without attention.

Disadvantages:

1. Expensive equipment is required.
2. Nonprotein nitrogen also is included.

15.2.10 Infrared Spectroscopy

15.2.10.1 Principle

Infrared spectroscopy measures the absorption of radiation (near- or mid-infrared regions) by molecules in food or other substances. Different functional groups in a food absorb different frequencies of radiation. For proteins and peptides, various mid-infrared bands (6.47 μm) and near-infrared (NIR) bands (e.g., 3300–3500 nm, 2080–2220 nm, 1560–1670 nm) characteristic of the peptide bond can be used to estimate the protein content of a food. By irradiating a sample with a wavelength of infrared light specific for the constituent to be measured, it is possible to predict the concentration of that constituent by measuring the energy that is reflected or transmitted by the sample (which is inversely proportional to the energy absorbed).

15.2.10.2 Procedure

See Chapter 27 for a detailed description of instrumentation, sample handling, and calibration and quantitation methodology.

15.2.10.3 Applications

Mid-infrared spectroscopy is used in Infrared Milk Analyzers to determine milk protein content, while near-infrared spectroscopy is applicable to a wide range of food products (e.g., grains; cereal, meat, and dairy products) (34-37). Instruments are expensive and they must be calibrated properly. However, samples can be analyzed rapidly (30 sec to 2 min) by analysts with minimal training.

15.3 COMPARISON OF METHODS

- Sample preparation: The Kjeldahl, Dumas, and infrared spectroscopy methods require little preparation. Sample particle size of 20 mesh or smaller generally is satisfactory for these methods. Some of the newer NIR instruments can make measurements directly on whole grains and other coarsely granulated products without grinding or other sample preparation. Other methods described in this chapter require fine particles for extraction of proteins from the complex food systems.
- Principle: The Dumas and Kjeldahl methods measure directly the total amount of organic nitrogen element in the foods; other methods measure the various properties of proteins. For instance, the biuret method measures peptide bonds, and the Lowry method measures a combination of peptide bonds and the amino acids tryptophan and tyrosine. Infrared spectroscopy is an indirect method to estimate protein content, based on the energy absorbed when a sample is subjected to a wavelength of infrared radiation specific for the peptide bond.
- Sensitivity: Kjeldahl, Dumas, biuret, and anionic dye binding methods are less sensitive than UV, Lowry, BCA, or Bradford methods.
- Speed: After the instrument has been properly calibrated, infrared spectroscopy is likely the most rapid of the methods discussed. In most other methods involving spectrophotometric (colorimetric) measurements, one must separate proteins from the interfering insoluble materials before mixing with the color reagents or must remove the insoluble materials from the colored protein-reagent complex after mixing. However, the speed of determination in the colorimetric methods and in the Dumas method is faster than with the Kjeldahl method.

15.4 SPECIAL CONSIDERATIONS

1. To select a particular method for a specific application, sensitivity, accuracy, and reproducibility as well as physicochemical properties of food materials must be considered. The data should be interpreted carefully to reflect what actually is being measured.

2. Food processing methods, such as heating, may reduce the extractability of proteins for analysis and cause an underestimation of the protein content measured by methods involving an extraction step (8).

3. All methods, except for the Dumas, Kjeldahl, and the UV method for purified proteins, require the use of a standard or reference protein or a calibration with the Kjeldahl method. In the methods using a standard protein, proteins in the samples are assumed to have similar composition and behavior compared to the standard protein. The selection of an appropriate standard for a specific type of food is important.

4. Nonprotein nitrogen is present in practically all foods. To determine protein nitrogen, the samples usually are extracted under alkaline conditions then precipitated with trichloroacetic acid or sulfosalicylic acid. The concentration of the acid used affects the precipitation yield. Therefore, nonprotein nitrogen content may vary with the type and concentration of the reagent used. Heating could be used to aid protein precipitation by acid, alcohol, or other organic solvents. In addition to acid precipitation methods used for nonprotein nitrogen determination, less empirical methods such as dialysis and ultrafiltration and column chromatography could be used to separate proteins from small nonprotein substances.

5. In the determination of the nutritive value of food proteins, including protein digestibility and protein efficiency ratio (PER), the Kjeldahl method with a 6.25 conversion factor is usually used to determine crude protein content. The PER could be underestimated if a substantial amount of nonprotein nitrogen is present in foods. A food sample with a higher nonprotein nitrogen content (particularly if the nonprotein nitrogen does not have many amino acids or small peptides) may have a lower PER than a food sample containing similar protein structure/composition and yet with a lower amount of nonprotein nitrogen.

15.5 SUMMARY

Methods based on the unique characteristics of proteins and amino acids have been described to determine the protein content of foods. The Dumas and Kjeldahl methods measure nitrogen. Copper-peptide bond interactions contribute to the analysis by the

biuret and Lowry methods. Amino acids are involved in the UV 280 nm, dye-binding, ninhydrin, and Lowry methods. The BCA method utilizes the reducing power of proteins in an alkaline solution. Infrared spectroscopy is based on absorption of a wavelength of infrared radiation specific for the peptide bond. The various methods differ in their speed and sensitivity.

In addition to the commonly used methods discussed, there are other methods available for protein quantification. Because of the complex nature of various food systems, problems may be encountered to different degrees in protein analysis by available methods. Rapid methods may be suitable for quality control purposes, while a sensitive method is required for work with a minute amount of protein. Indirect colorimetric methods usually require the use of a carefully selected protein standard or a calibration with an official method (e.g., Kjeldahl).

15.6 STUDY QUESTIONS

1. What factors should one consider when choosing a method for protein determination?.
2. The Kjeldahl method of protein analysis consists of three major steps. List these steps in the order they are done, and describe in words what occurs in each step. Make it clear why milliliters of HCl can be used as an indirect measure of the protein content of a sample.
3. Why is the conversion factor from Kjeldahl nitrogen to protein different for various foods, and how is the factor of 6.25 obtained?
4. How can Nesslerization or the procedure that uses phenol and hypochlorite be used as part of the Kjeldahl procedure, and why might they best be put to use?
5. Differentiate and explain the chemical basis of the following techniques that can be used to quantitate proteins in quality control/research:
   a. Kjeldahl method
   b. turbidimetric method
   c. ninhydrin method
   d. absorbance at 280 nm
   e. absorbance at 220 nm
   f. biuret method
   g. Lowry method
   h. Bradford method
   i. bicinchoninic acid method
   j. Dumas method
   k. infrared spectroscopy
6. Differentiate the principles of protein determination by dye binding with an anionic dye such as Amido Black versus with the Bradford method, which uses the dye Coomassie Brilliant Blue G-250.
7. With the anionic dye binding method, would a sample with a higher protein content have a higher or a lower absorbance reading than a sample with a low protein content? Explain your answer.
8. For each of the situations described below, identify a protein assay method most appropriate for use, and indicate

the chemical basis of the method (i.e., what does it really measure?).
   a. nutrition labeling
   b. intact protein eluting from a chromatography column; qualitative or semiquantitative method
   c. intact protein eluting from a chromatography column; colorimetric, quantitative method
   d. amino acids eluting from an ion-exchange chromatography column; quantitative method
   e. rapid, quality control method for protein content of cereal grains

15.7 PRACTICE PROBLEMS

1. A dehydrated precooked pinto bean was analyzed for crude protein content in duplicate using the Kjeldahl method. The following data were recorded:
   moisture content = 8.0%.
   wt of sample no. 1 = 1.015 g
   wt of sample no. 2 = 1.025 g.
   normality of HCl used for titration = 0.1142 ml
   HCl used for sample no. 1 = 22.0 ml.
   HCl used for sample no. 2 = 22.5 ml
   HCl used for reagent blank = 0.2ml.

Calculate crude protein content on both wet and dry weight basis of the pinto bean, assuming pinto bean protein contains 17.5% nitrogen.

2. A 10-ml protein fraction recovered from a column chromatography was analyzed for protein using the BCA method. The following data were obtained from a duplicate analysis using BSA as a standard:

BSA mg/ml	Mean Absorbance at 562 nm
0.2	0.25
0.4	0.53
0.6	0.74
0.8	0.92
1.0	1.20

The average absorbance of a 0.5-ml sample was 0.43. Calculate protein content (mg/ml) and total protein quantity of this column fraction.

Answers

1. Protein content = 19.8% on a wet weight basis; 21.4% on a dry weight basis.
2. Protein content = 0.68 mg/ml. Total protein quantity = 6.8 mg.

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Protein Separation and Characterization Procedures

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16.1 INTRODUCTION

Many protein separation techniques are available to food scientists. Several of the separation techniques described in this chapter are used commercially for the production of food or food ingredients, whereas others are used to purify a protein from a food for further study in the laboratory. In general, separation techniques exploit the biochemical differences in protein solubility, size, charge, adsorption characteristics, and biological affinities for other molecules. These physical characteristics are then used to purify individual proteins from complex mixtures. Some techniques to characterize the biochemical properties of a protein are also presented in this chapter.

16.2 INITIAL CONSIDERATIONS

Usually, several separation techniques are used in sequence to purify a protein from a food. In general, the more separation steps used, the higher the purity of the resulting preparation. Food ingredients such as protein concentrates may be prepared using only one separation step because high purity is not necessary. To prepare a pure protein for laboratory study it is often necessary to use three or more separation steps in sequence to achieve a highly purified protein preparation.

Before starting a separation sequence, it is necessary to learn as much as possible about the biochemical properties of a protein, such as molecular weight, isoelectric point (pI), solubility properties, and denaturation temperature, to determine any unusual physical characteristics that will make separation easier. The first separation step should be one that can easily be used with large quantities of material. This is often a technique that utilizes the differential solubility properties of a protein. Each succeeding step in a purification sequence will use a different mode of separation. The most common methods of purification include precipitation, ion-exchange chromatography, affinity chromatography, and size-exclusion chromatography (1).

16.3 METHODS OF PROTEIN SEPARATION

16.3.1 Separation By Differential Solubility Characteristics

16.3.1.1 Principle

Separation by precipitation exploits the differential solubility properties of proteins in solution. Proteins are polyelectrolytes; thus, solubility characteristics are determined by the type and charge of amino acids in the molecule. Proteins can be selectively precipitated

or solubilized by changing buffer pH, ionic strength, dielectric constant, or temperature. These separation techniques are advantageous when working with large quantities of material, are relatively quick, and are not usually influenced by other food components. Precipitation techniques are used most commonly during early stages of a purification sequence.

16.3.1.2 Procedures

16.3.1.2.1 Salting Out Proteins have unique solubility profiles in neutral salt solutions. Low concentrations of neutral salts usually increase the solubility of proteins; however, proteins are precipitated from solution as ionic strength is increased. This property can be used to precipitate a protein from a complex mixture. Ammonium sulfate $[(NH_4)_2SO_4]$ is commonly used because it is highly soluble, although other neutral salts such as NaCl or KCl may be used to salt out proteins. Generally a two-step procedure is used to maximize separation efficiency. In the first step, $(NH_4)_2SO_4$ is added at a concentration just below that necessary to precipitate the protein of interest. When the solution is centrifuged, less soluble proteins are precipitated while the protein of interest remains in solution. The second step is performed at an $(NH_4)_2SO_4$ concentration just above that necessary to precipitate the protein of interest. When the solution is centrifuged, the protein is precipitated, while more soluble proteins remain in the supernatant. One disadvantage of this method is that large quantities of salt contaminate the precipitated protein and often must be removed before the protein is resolubilized in buffer. Tables and formulas are available in many biochemistry textbooks for calculating the proper amount of $(NH_4)_2SO_4$ to achieve a specific concentration (2).

16.3.1.2.2 Isoelectric Precipitation The isoelectric point (pI) is defined as the pH at which a protein has no net charge in solution. Proteins aggregate and precipitate at their pI because there is no electrostatic repulsion between molecules. Proteins have different pIs; thus, they can be separated from each other by adjusting solution pH. When the pH of a solution is adjusted to the pI of a protein, the protein precipitates while proteins with different pIs remain in solution. The precipitated protein can be resolubilized in another solution of different pH.

16.3.1.2.3 Solvent Fractionation Protein solubility at a fixed pH and ionic strength is a function of the dielectric constant of a solution. Thus, proteins can be separated based on solubility differences in organic solvent–water mixtures. The addition of water-miscible organic solvents, such as ethanol or acetone, decreases the dielectric constant of an aqueous solution and

decreases the solubility of most proteins. Organic solvents decrease ionization of charged amino acids, resulting in protein aggregation and precipitation. The optimum quantity of organic solvent to precipitate a protein varies from 5% to 60%. Solvent fractionation is usually performed at 0°C or below to prevent protein denaturation caused by temperature increases that occur when organic solvents are mixed with water.

16.3.1.2.4 Denaturation of Contaminating Proteins

Many proteins are denatured and precipitated from solution when heated above a certain temperature or by adjusting a solution to highly acid or basic pHs. Proteins that are stable at high temperatures or at extremes of pH are most easily separated by this technique because many contaminating proteins can be precipitated while the protein of interest remains in solution.

16.3.1.3 Applications

All of the above techniques are commonly used to fractionate proteins. The differential solubility of selected muscle proteins in $(NH_4)_2SO_4$ and acetone and temperature stability at 55°C are illustrated in Table 16-1. These three techniques can be combined in sequence to prepare muscle proteins of high purity.

One of the best examples of the commercial use of differential solubility to separate proteins is in production of protein concentrates. Soy protein concentrate can be prepared from defatted soybean flakes or flour using several methods described previously. Soy proteins can be precipitated from other soluble constituents in the flakes or flour using a 60–80% aqueous alcohol solution, by isoelectric precipitation at pH 4.5

(which is the pI of many soy proteins), or by denaturation with moist heat. These methods have been used to produce concentrates containing greater than 65% protein. Two or three separation techniques can be combined in sequence to produce soy protein isolates with protein concentrations above 90%.

16.3.2 Separation by Adsorption

16.3.2.1 Principle

Adsorption chromatography is defined as the separation of compounds by adsorption to, or desorption from, the surface of a solid support by an eluting solvent. Separation is based on differential affinity of the protein for the adsorbent or eluting buffer. Affinity chromatography and ion-exchange chromatography are two types of adsorption chromatography that will be described briefly below (see Chapter 31 for a more detailed description).

16.3.2.2 Procedures

16.3.2.2.1 Ion-Exchange Chromatography Ion-exchange chromatography is defined as the reversible adsorption between charged molecules and ions in solution and a charged solid support matrix. Ion-exchange chromatography is the most commonly used protein separation technique and results in an average eightfold purification (1). A positively charged matrix is called an anion-exchanger because it binds negatively charged ions or molecules in solution. A negatively charged matrix is called a cation-exchanger because it binds positively charged ions or molecules. The most commonly used exchangers for protein purification are anionic diethylaminoethyl derivatized

16-1 table Conditions for Fractionating Water-Soluble Muscle Proteins Using Differential Solubility Techniques

Enzyme	(NH₄)₂SO₄ pH 5.5, 10°C (Percent Saturation)	Acetone pH 6.5, -5°C (Percent vol/vol)	Stability pH 5.5, 55°C
Phosphorylase	30–40	16–20	U
Pyruvate kinase	55–65	25–40	S
Aldolase	45–55	30–40	S
Lactate dehydrogenase	50–60	25–35	S
Enolase	60–70	35–45	U
Creatine kinase	60–80	35–45	U
Phosphoglycerate kinase	60–75	45–60	S
Myoglobin	70–80	45–60	U

Adapted from (3) with permission of the University of Wisconsin Press. From Briskey, E.J., G. Cassens, and B.B. Marsh The Physiology and Biochemistry of Muscle as a Food. Copyright 1970
U = unstable and S = stable at heating temperature

supports, followed by carboxymethyl and phospho cation-exchangers (see Chapter 31).

The protein of interest is first adsorbed to the ion exchanger under buffer conditions (ionic strength and pH) that maximize the affinity of the protein for the matrix. Contaminating proteins of different charges pass through the exchanger unabsorbed. Proteins bound to the exchangers are selectively eluted from the column by gradually changing the ionic strength or pH of the eluting solution (Fig. 16-1). As the composition of the eluting buffer changes, the charges of the proteins change and their affinity for the ion-exchange matrix is decreased.

16.3.2.2.2 Affinity Chromatography Affinity chromatography is a type of adsorption chromatography in which a protein is separated in a chromatographic matrix containing a ligand covalently bound to a solid support. A ligand is defined as a molecule with a reversible, specific, and unique binding affinity for a protein. Ligands include enzyme inhibitors, enzyme substrates, coenzymes, antibodies, and certain dyes. Covalently bound ligands can be purchased commercially or prepared in the laboratory.

The protein is passed through a column containing the ligand bound to a solid support, under buffer conditions (pH, ionic strength, temperature, and protein concentration) that maximize binding of the protein to the ligand. Contaminating proteins and molecules that do not bind the ligand are eluted. The bound protein is then desorbed or eluted from the column under conditions that decrease the affinity of the protein for the bound ligand, by changing the pH, temperature, or concentration of salt or ligand in the eluting buffer.
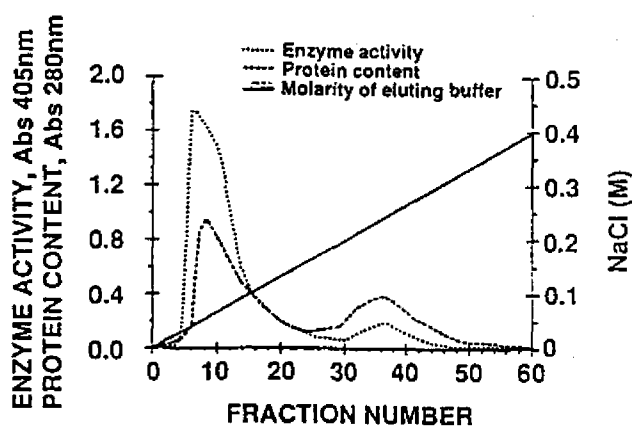
Affinity chromatography is a very powerful technique and is the second most commonly used protein purification procedure (1). The average purification achieved by affinity chromatography is approximately 100-fold, although 1000-fold increases in purification have been reported. This technique is more powerful than size-exclusion, ion-exchange, and other separation methods that usually achieve less than a 12-fold purification. The development of new affinity chromatography procedures can be very time consuming because many variables must be optimized, which is a major disadvantage to the method. Also, the affinity materials are often more expensive than other separation media.

16.3.2.2.3 High Performance Liquid Chromatography Many chromatographic methods have been adapted for use with high performance liquid chromatography (HPLC) systems. The use of HPLC to separate proteins was made possible by development of macroporous, microparticulate packing materials that withstand high pressures. This technique is discussed in more detail in Chapter 32.

16.3.2.3 Applications

Ion-exchange chromatography is commonly used to separate proteins in the laboratory and can be used for quantification of amino acids in a protein as described in section 16.3.5. Affinity chromatography has many uses in the analytical lab, and may be used for commercial preparation of protein reagents by chemical suppliers, but is not generally used for commercial production of food protein ingredients due to the high costs involved.

Affinity chromatography is used to purify many glycoproteins. Glycoproteins can be separated from other proteins in a complex mixture by utilization of the high carbohydrate binding affinity of lectins. Lectins, such as concanavalin A, are carbohydrate-binding proteins that can be bound to a solid support and used to bind the carbohydrate moiety of glycoproteins that are applied to the column. Once the glycoproteins are bound to the column, they can be desorbed using an eluting buffer containing an excess of lectin. The glycoproteins bind preferentially to the free lectins and elute from the column.

16.3.3 Separation by Size

16.3.3.1 Principle

Protein molecular weights range from about 10,000 to over 1,000,000; thus, size is a logical parameter to exploit for separations. Actual separation occurs based

16-1
figure

Elution profile of two α-galactosidase isozymes from a carboxymethyl-cellulose ion-exchange column. (Adapted from *Phytochemistry*, 28, S.R. Alani, D.M. Smith, and P. Markakis, α-Galactosidases of *Vigna unquiculata*, p. 2058, Copyright 1989, with kind permission from Elsevier Science, Kidlington, England.)

on the Stokes radius of the protein, not on the molecular weight. Stokes radius is the average radius of the protein in solution and is determined by protein conformation. For example, a globular protein may have an actual radius very similar to its Stokes radius, whereas a fibrous or rod-shaped protein of the same molecular weight may have a Stokes radius that is much larger than that of the globular protein. As a result, the two proteins may separate as if they had different molecular weights.

16.3.3.2 Procedures

16.3.3.2.1 Dialysis Dialysis is used to separate molecules in solution by the use of semipermeable membranes that permit passage of small molecules but not larger molecules. To perform dialysis, a protein solution is placed into dialysis tubing that has been tied or clamped at one end. The other end of the tubing is sealed, and the bag is placed in a large volume of water or buffer (usually 500–1000 times greater than the sample volume inside the dialysis tubing) which is slowly stirred. Low-molecular-weight solutes diffuse from the bag, while buffer diffuses into the bag. Dialysis is simple; however, it is a relatively slow method, usually requiring at least 12 hr and one change of buffer. The protein solution inside the bag is often diluted during dialysis, due to osmotic strength differences between the solution and dialysis buffer.

This technique can be used to concentrate protein by coating the dialysis bag containing a protein solution with polyethylene glycol. Polyethylene glycol absorbs water and concentrates the solution within the dialysis bag. Equilibrium dialysis can be used for determining the stoichiometry of protein–ligand binding.

16.3.3.2.2 Ultrafiltration Ultrafiltration is a technique using a semipermeable membrane for the separation of solutes on the basis of size under an applied pressure. This method is similar to dialysis but is much faster. Semipermeable membranes with molecular weight cutoffs from 500 to 300,000 are available. Molecules larger than the membrane cut-off are retained and become part of the retentate, while smaller molecules pass through the membrane and become part of the ultrafiltrate. Ultrafiltration can be used to concentrate a protein solution, remove salts, exchange buffer, or fractionate proteins on the basis of size.

Several types of laboratory and production scale ultrafilter are commercially available. A stirred cell ultrafiltration unit is illustrated in Fig. 16-2. The protein solution in the stirred cell is filtered through the semipermeable membrane by gas pressure, leaving a concentrated solution of proteins larger than the mem-
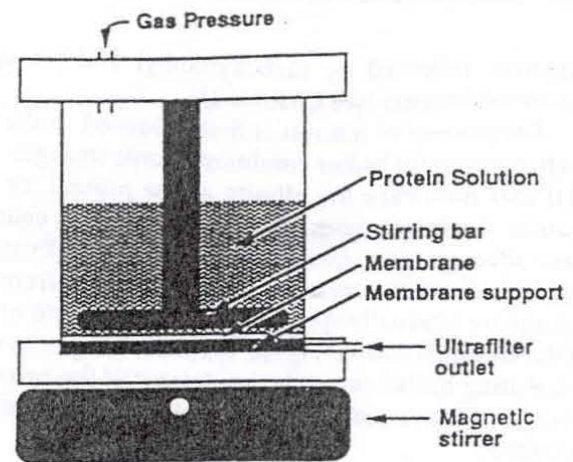
16-2 figure Schematic diagram of a stirred cell ultrafiltration unit.

brane cutoff point inside the cell. Some ultrafiltration devices are designed for use in a centrifuge.

16.3.3.2.3 Size-Exclusion Chromatography Size-exclusion chromatography, also known as gel filtration or gel permeation chromatography, is a column technique that can be used to separate proteins on the basis of size. A protein solution is allowed to flow down a column packed with a solid support of porous beads made of a cross-linked polymeric material such as agarose or dextran. Molecules larger than the pores in the beads are excluded, moving quickly through the column and eluting from the column in the shortest times. Small molecules enter the pores of the beads and are retarded, thus moving very slowly through the column. Molecules of intermediate sizes partially interact with the porous beads and elute at intermediate times. Consequently, molecules are eluted from the column in order of decreasing size.

Beads of different average pore sizes that allow for efficient fractionation of proteins of different molecular weights are commercially available. Chemical suppliers list working molecular weight ranges for each of their gel permeation solid support products. Size-exclusion chromatography is used to remove salts, change buffers, fractionate proteins, and estimate molecular weight. Molecular weight can be calculated by chromatographing the unknown protein and several proteins of known molecular weights. Standards of known molecular weight are commercially available and can be used to prepare a standard curve. A plot of the elution volume (V_e) of each protein versus log of the molecular weight yields a straight line. Size-exclusion techniques generally can be used to estimate molecular weights within $\pm 10\%$; however, errors can occur if the Stokes radii of the unknown protein and stan-

dards are quite different. More information on size-exclusion chromatography is available in Chapter 31.

16.3.3.3 Applications

Dialysis and size-exclusion chromatography are primarily used in the analytical laboratory in a protein separation sequence. Dialysis is commonly used to change the buffer to one of the appropriate pH and ionic strength prior to electrophoresis of a protein sample. Dialysis is usually performed after $(NH_4)_2SO_4$ precipitation of a protein to remove excess salt and other small molecules and to solubilize protein in a new buffer.

Ultrafiltration is used both in the laboratory and for commercial applications. Ultrafiltration is commonly used for the preparation of protein concentrates from whey, which is a by-product of the cheesemaking industry. In this process, a semipermeable ultrafiltration membrane with a molecular weight cutoff of 10,000–20,000 is used to partially remove lactose, salts, and water from whey and concentrate proteins in the retentate (5).

16.3.4 Separation by Electrophoresis

16.3.4.1 Polyacrylamide Gel Electrophoresis

16.3.4.1.1 Principle
Electrophoresis is defined as the migration of charged molecules in a solution through an electrical field. The most common type of electrophoresis performed with proteins is zonal electrophoresis in which proteins are separated from a complex mixture into bands by migration in aqueous buffers through a solid polymer matrix called a gel. Polyacrylamide gels are the most common matrix for zonal electrophoresis of proteins, although other matrices such as starch and agarose may be used. Gel matrices can be formed in glass tubes or as slabs between two glass plates.

Separation depends on the friction of the protein within the matrix and the charge of the protein molecule as described by the following equation:

$$\text{Mobility} = \frac{(\text{applied voltage})(\text{net charge on molecule})}{\text{friction of the molecule}} \quad [1]$$

Proteins are positively or negatively charged, depending on solution pH and their pI. A protein is negatively charged if solution pH is above its pI, whereas a protein is positively charged if solution pH is below its pI. The magnitude of the charge and applied voltage will determine how far a protein will migrate in an electrical field. The higher the voltage and stronger the charge on the protein, the greater the migration within the electrical field. Molecular size and shape, which determine the Stokes radius of a protein, also determine migration distance within the gel matrix.

Mobility of proteins decreases as molecular friction increases due to an increase in Stokes radius; thus, smaller proteins tend to migrate faster through the gel matrix. Similarly, a decrease in pore size of the gel matrix will decrease mobility.

In nondenaturing or native electrophoresis, proteins are separated in their native from based on charge, size, and shape of the molecule. Another form of electrophoresis commonly used for separating proteins is denaturing electrophoresis (6). Polyacrylamide gel electrophoresis (PAGE) with an anionic detergent, sodium dodecyl sulfate (SDS), is used to separate protein subunits by size. Proteins are solubilized and dissociated into subunits in a buffer containing SDS and a reducing agent. Reducing agents, such as mercaptoethanol or dithiothreitol, are used to reduce disulfide bonds within a protein subunit or between subunits. Proteins bind SDS, become negatively charged, and are separated based on size alone.

16.3.4.1.2 Procedures
A power supply and electrophoresis apparatus containing the polyacrylamide gel matrix and two buffer reservoirs are necessary to perform a separation. A representative slab gel and electrophoresis unit is shown in Fig. 16-3. The power supply is used to make the electric field by providing a source of constant current, voltage, or power. The electrode buffer controls the pH to maintain the proper charge on the protein and conducts the current through the polyacrylamide gel. Commonly used buffer systems include an anionic tris-(hydroxymethyl)aminomethane buffer with a resolving gel at pH 8.8 (7) and a cationic acetate buffer at pH 4.3 (8).

The polyacrylamide gel matrix is formed by polymerizing acrylamide and a small quantity (usually 5%

Sample well | Electrode Buffer, pH 8.3

Upper Buffer Reservoir

Stacking Gel, pH 6.8

Direction of Protein Migration

Resolving Gel, pH 8.8

Lower Buffer Reservoir

Electrode Buffer, pH 8.3
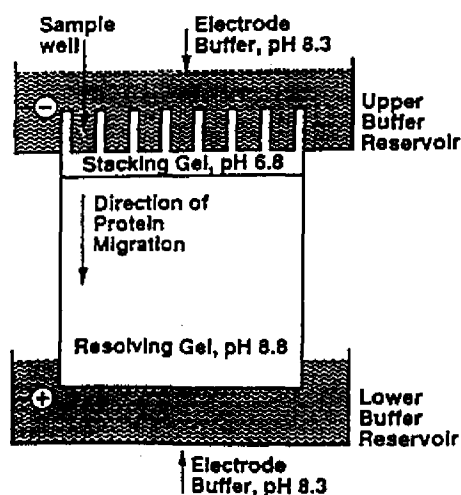
16-3 figure Schematic diagram of a slab gel electrophoresis unit indicating the pHs of the stacking and resolving gels and the electrode buffer in an anionic discontinuous buffer system.

or less) of the cross-linking reagent, N,N'-methyl-enebisacrylamide, in the presence of a catalyst, tetramethylethylenediamine (TEMED), and source of free radicals, ammonium persulfate, as illustrated Fig. 16-4. Gels can be made in the laboratory or purchased precast.

A discontinuous gel matrix is usually used to improve resolution of proteins within a complex mixture (9,10). The discontinuous matrix consists of a stacking gel with a large pore size (usually 3–4% acrylamide) and a resolving gel of a smaller pore size. The stacking gel, as its name implies, is used to stack or concentrate the proteins into very narrow bands prior to their entry into the resolving gel. At pH 6.8, a voltage gradient is formed between the chloride (high negative charge) and glycine ions (low negative charge) in the electrode buffer, which serves to stack the proteins into narrow bands between the ions. Migration into the resolving gel of a different pH disrupts this voltage gradient and allows separation of the proteins into discrete bands.

The pore size of the resolving gel is selected based on the molecular weight of the proteins of interest and is varied by altering the concentration of acrylamide in solution. Proteins are usually separated on resolving gels that contain 4–15% acrylamide. Acrylamide concentrations of 15% are often used to separate proteins with molecular weights below 50,000. Proteins greater than 500,000 daltons are often separated on gels with acrylamide concentrations below 7%. A gradient gel in which the acrylamide concentration increases from the top to the bottom of the gel is often used to separate a mixture of proteins with a large molecular weight range.

To perform a separation, proteins in a buffer of the appropriate pH are loaded on top of the stacking gel. Bromophenol blue tracking dye is added to the protein solution. This dye is a small molecule that migrates

ahead of the proteins and is used to monitor the progress of a separation. After an electrophoresis run, the bands on the gels are generally visualized using a protein stain such as Coomassie Brilliant Blue or silver stain. Specific enzyme stains or antibodies can be used to detect a protein.

The electrophoretic or relative mobility (R_m) of each protein band is calculated as:

$$R_m = \frac{\text{distance protein migrated from start of resolving gel}}{\text{distance between start of running gel and tracking dye}} \quad [2]$$

16.3.4.1.3 Applications Electrophoresis is often used to determine the protein composition of a food product. For example, differences in the protein composition of soy protein concentrates and whey protein concentrates produced by different separation techniques can be detected. Electrophoresis can also be used to determine the purity of a protein extract.

SDS–PAGE is used to determine subunit composition of a protein and to estimate subunit molecular weight within an error of ±5%, although highly charged proteins or glycoproteins may be subject to a larger error. Molecular weight is determined by comparing R_m of the protein subunit with R_m of protein standards of known molecular weight (Fig. 16-5). Commercially prepared protein standards are available in several molecular weight ranges. To prepare a standard curve, logarithms of protein standard molecular weights are plotted against their corresponding R_m values. The molecular weight of the unknown protein is determined from its R_m value using the standard curve.

16.3.4.2 Isoelectric Focusing

16.3.4.2.1 Principle Isoelectric focusing is a modification of electrophoresis, in which proteins are separated by charge in an electric field on a gel matrix in which a pH gradient has been generated using ampholytes. Proteins are focused or migrate to the location in the gradient at which pH equals the pI of the protein. Resolution is among the highest of any protein separation technique and can be used to separate proteins with pIs that vary less than 0.02 of a pH unit.

Acrylamide   N,N'-methylene-   Polymer
             bis-acrylamide

16-4
figure

Free radical polymerization reaction of polyacrylamide.

16.3.4.2.2 Procedure A pH gradient is formed using ampholytes, which are small polymers (molecular mass of about 5000 daltons) containing both positively and negatively charged groups. An ampholyte mixture is composed of thousands of polymers that exhibit a range of pK values. Ampholytes are added to the gel solution prior to polymerization. After the gel is formed and a current applied, the ampholytes migrate to produce the pH gradient; negatively charged

(A)

(B)

16-5 figure Use of SDS-PAGE to determine the molecular weight of a protein. (A) Separation of molecular weight standards and the unknown protein. (B) Standard curve for estimating protein molecular weight.

ampholytes migrate toward the anode while positively charged ampholytes migrate toward the cathode. Ampholyte mixtures are available that cover a narrow pH range (2–3 units) or a broad range (pH 3–10) and should be selected for use based on properties of the proteins to be separated.

16.3.4.2.3 Applications Isoelectric focusing is the method of choice for determining the isoelectric point of a protein and is an excellent method for determining the purity of a protein preparation. For example, isozymes of polyphenol oxidase and other plant and animal proteins are identified using isoelectric focusing. Isoelectric focusing is used to differentiate closely related fish species based on protein patterns.

Isoelectric focusing and SDS-PAGE can be combined to produce a two-dimensional electrophoretogram that is extremely useful for separating very complex mixtures of proteins. This technique is called two-dimensional electrophoresis (11). Proteins are first separated in tube gels by isoelectric focusing. The tube gel containing the separated proteins is then placed on top of an SDS-PAGE slab gel, and proteins are separated. Thus, proteins are separated first on the basis of charge and then according to size and shape. Over 1000 proteins in a complex mixture have been resolved using this technique.

## 16.3.4.3 Capillary Electrophoresis

16.3.4.3.1 Principle Similar principles apply for the separation of proteins by both capillary and conventional electrophoretic techniques; proteins can be separated on the basis of charge or size in an electric field. The primary difference between capillary electrophoresis and conventional electrophoresis (described previ-

ously) is that capillary tubing is used in place of acrylamide gels cast in tubes or slabs. Electroosmotic flow within the capillary also can influence separation of proteins in capillary electrophoresis and is discussed briefly in section 16.3.4.3.3 (12).

16.3.4.3.2 Procedure A schematic diagram of a capillary electrophoresis system is shown in Fig. 16-6. A capillary electrophoresis system is comprised of a capillary column, power supply, detector, and two buffer reservoirs. The sample is introduced into the inlet side of the capillary tube by simply replacing the inlet buffer reservoir with the sample solution and applying low pressure or voltage across the capillary until the desired volume of sample has been loaded onto the column. Capillaries are composed of fused silica with internal diameters that commonly range from 25 to 100 µm. Column length varies from a few centimeters to 100 cm. High electric fields (100–500 V/cm) can be used as the narrow columns dissipate heat very effectively, allowing for short run times of 10–30 min.

At the end of a run protein bands are not visualized by staining as in conventional electrophoresis. Instead, protein bands are detected on the column as they migrate past a detector. The detectors are similar to those used in high performance liquid chromatography described in Chapter 32. UV-visible detectors are most common, although fluorescence and conductivity detectors are available. The data obtained from a capillary electrophoresis run look like a typical chromatogram from a gas chromatograph or high performance liquid chromatograph (Fig. 16-7).

15.3.4.3.3 Applications Capillary electrophoresis is an emerging technique still used primarily in analytical labs and not for routine quality control purposes.
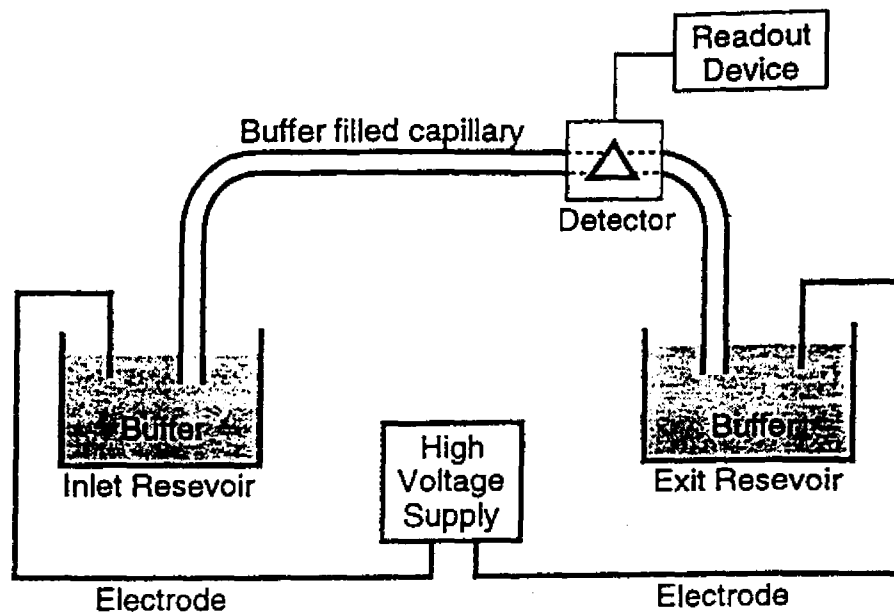
16-6
figure

Schematic diagram of a capillary electrophoresis system.
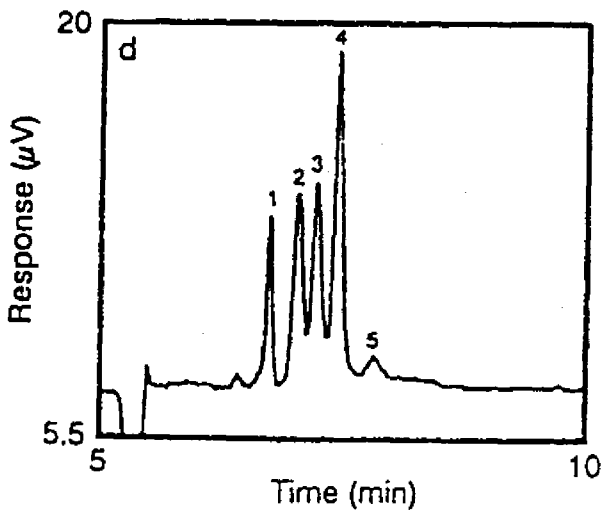
16-7
figure

Electropherogram from a capillary zone electrophoresis run showing the separation of several proteins from whey (72 cm × 50 μm column, 20 kV, 50 mM MES buffer, pH 8.0. 1. α-lactalbumin, 2. β-lactoglobulin C, 3. β-lactoglobulin B, 4. β-lactoglobulin A, 5. bovine serum albumin. [Adapted from (13) with permission.]

There are three variations of capillary electrophoresis commonly used for protein separations.

Capillary zone electrophoresis or free solution electrophoresis is much like native polyacrylamide gel electrophoresis, except proteins are separated in free solution inside capillary tubes filled with buffer of the desired pH. Diffusion is prevented within the narrow

diameter of the capillaries so a gel matrix is not required. In capillary zone electrophoresis, electroosmotic flow also influences the separation of proteins within capillary tubes. The negatively charged fused silica capillary wall [containing silanol groups (SiO⁻)] attracts positively charged ions (cations) from the buffer to form a double ion layer at the interface between the capillary column wall and the buffer. When the electric field is applied, the cations forming the double layer are attracted toward the cathode and "pull" other molecules (independent of charge) in the same direction. Thus, in free solution capillary electrophoresis, cations, anions, and uncharged molecules can be separated in a single run. Electroosmotic flow can be controlled by changing the pH or ionic strength of the buffer to alter the charge on the capillary wall and change the rate of protein migration. Capillary zone electrophoresis has been used to fractionate milk proteins (13), soy proteins (14), and cereal proteins (15).

SDS capillary gel electrophoresis techniques can be used to separate proteins by size to determine molecular masses. In this technique proteins are denatured and dissociated in the presence of SDS and a reducing agent, then fractionation occurs in polyacrylamide gel-filled capillaries of specific pore sizes. Alternatively, linear polymers, such as methyl cellulose, dextrans, or polyethylene glycol, are added to the buffer within the capillary in a technique called dynamic sieving capillary electrophoresis. These entangled polymers act like the pores of the polyacrylamide gel to slow migration of the larger proteins and allow separation by size.

Proteins also can be separated on the basis of their

isoelectric points, in a technique called capillary iso-electric focusing. Ampholytes (described in section 16.3.4.2.2) are used to form a pH gradient within the capillary tube. A gel matix is not needed. In this technique, electroosmotic flow is minimized by coating the capillary walls with buffer additives to prevent unde-sirable effects caused by surface charge.

16.3.5 Amino Acid Analysis

16.3.5.1 Principle

Amino acid analysis is used to quantitatively deter-mine the amino acid composition of a protein. The pro-tein sample is first hydrolyzed to release the amino acids. Amino acids are then separated using chromato-graphic techniques and quantified. Ion-exchange chro-matography, reversed-phase liquid chromatography, and gas-liquid chromatography are three separation techniques used. This section will describe the use of ion-exchange and reversed-phase liquid chromatogra-phy.

16.3.5.2 Procedures

In general, a protein sample is hydrolyzed in constant boiling 6 N HCl for 24 hr to release amino acids prior to chromatography. Accurate quantification of some amino acids is difficult because they react differently during hydrolysis. Consequently, special hydrolysis procedures must be used to prevent errors.

Tryptophan is completely destroyed by acid hydrolysis. Methionine, cysteine, threonine, and serine are progressively destroyed during hydrolysis; thus, the duration of hydrolysis will influence results. Asparagine and glutamine are quantitatively con-verted to aspartic and glutamic acid, respectively, and cannot be measured. Isoleucine and valine are hydrolyzed more slowly in 6 N HCl than other amino acids, while tyrosine may be oxidized.

In general, losses of threonine and serine can be estimated by hydrolysis of samples for three periods of time (i.e., 24, 48, and 72 hr) followed by amino acid analysis. Compensation for amino acid destruction may be made by calculation to zero time assuming first-order kinetics. Valine and isoleucine are often esti-mated from a 72-hr hydrolysate. Cysteine and cystine can be converted to the more stable compound, cysteic acid, by hydrolysis in performic acid and then hydrolyzed in 6 M HCl and chromatographed. Tryptophan can be separated chromatographically after a basic hydrolysis or analyzed using a method other than amino acid analysis.

In the original method developed by Moore and colleagues (16) and later revised by Stein et al. (17), amino acids were separated by ion-exchange chro-matography using a stepwise elution with buffers of increasing pH and ionic strength. Amino acids eluting from the column were quantified by reaction with nin-hydrin to produce a colored product that was mea-sured spectrophotometrically. This method was auto-mated in the late 1970s and is the basis of many amino acid analysis systems in use today. It was adapted for use with high performance liquid chromatographs in the 1980s. This adaptation was made possible because new ion-exchange resins were developed that could withstand high pressures and extremes of pH, ionic strength, and temperature.

Other methods were also developed in the 1980s using HPLC and a reversed-phase column. The hydro-lyzed amino acids are derivatized prior to chromatog-raphy with phenylthiocarbamyl or other compound, separated by reversed-phase HPLC, and quantified by ultraviolet (UV) spectroscopy. Methods using HPLC techniques can detect picomole quantities of amino acids. Chromatographic runs usually take 30 min or less. A chromatogram showing the separation of amino acids in an infant formula is shown in Fig. 16-8.

The quantity of each amino acid in a peak is usu-ally determined by spiking the sample with a known quantity of internal standard. The internal standard is usually an amino acid, such as norleucine, not com-monly found in a food product. Results are usually expressed as mole percent. This quantity is calculated by dividing the mass of each amino acid (determined from the chromatogram) by its molecular weight, sum-ming the values for all amino acids, dividing each by the total moles, and multiplying the result by 100.

16.3.5.3 Applications

Amino acid analysis is used to determine the nutri-tional quality of a protein and to characterize or iden-tify newly isolated protein. Amino acid analysis pro-vides information for calculating the molecular weight of a protein as well as its partial specific volume. Proteins used in animal diets, infant formulas, and spe-cial human diets are often analyzed for protein quality to ensure adequate quantities of essential amino acids.

16.4 PROTEIN VISUALIZATION BY MICROSCOPY

While quantitating or separating proteins may be the objective in many situations, it may be necessary at times to visualize the location of protein molecules within foods or food ingredients. Fluorescence microscopy with stains specific to proteins can be applied to this problem (19–21). For example, the dye 1-anilino 8-naphthalene sulfonic acid (ANS) fluoresces only when bound to protein. An aqueous solution of the dye is reacted with the protein-containing sample, and the preparation viewed under a fluorescence
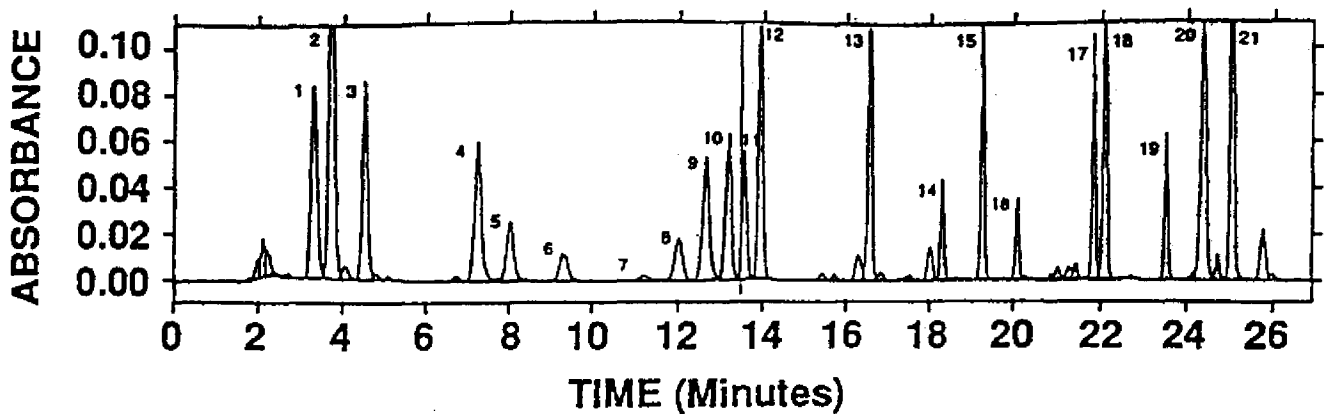
High performance liquid chromatographic analysis of phenylthiocarbamyl derived amino acids from infant formula separated on a reversed-phase column. Sample was spiked with taurine (1. Asp, 2. Glu, 3. internal standard, 4. Ser, 5. Gly, 6. His, 7. Tau, 8. Arg, 9. Thr, 10. Ala, 11. NH$_3$, 12. Pro, 13. internal standard, 14. Tyr, 15. Val, 16. Met, 17. Ile, 18. Leu, 19. Phe, 20. reagent, 21. Lys). [Adapted from (18), with permission from Millipore Corporation, © 1990 by Millipore Corporation.]

microscope. Other dyes used to visualize proteins are Coomassie Brilliant Blue and Fast Green. Staining intensity is influenced by compositional differences in the protein and by structural changes due to processing. Example applications include visualizing the distribution of proteins in cereal products, cheeses, and chocolates.

16.5 SUMMARY

This chapter has provided a brief introduction into a few techniques used to separate and characterize proteins that rely on the differences between protein molecules in their solubility, size, charge, adsorption characteristics, and biological affinity for other molecules. More detailed information on these techniques can be found in other publications (2,22-24).

16.6 STUDY QUESTIONS

1. For each of the techniques listed below, identify the basis by which it can be used to separate proteins within a protein solution (e.g., precipitation, adsorption, size, charge) and give a brief explanation of how/why it works in that way.
   a. dialysis
   b. adjustment of pH to pI
   c. addition of ammonium sulfate
   d. ultrafiltration
   e. heating to high temperature
   f. addition of ethanol
   g. affinity chromatography
   h. size-exlusion chromatography
2. Compare and contrast the principles and procedures of SDS-PAGE versus isoelectric focusing to separate proteins. Include in your explanation how and why it is possible to separate proteins by each method and what you

can learn about the protein by running it on each type of system.

3. Explain how capillary electrophoresis differs from SDS-PAGE.
4. You are submitting a soy protein sample to a testing laboratory with an amino acid analyzer (ion-exchange chromatography) so that you can obtain the amino acid composition. Explain how (a) the sample will be treated initially and (b) the amino acids will be quantified as they elute from the ion-exchange column. Describe the procedures. Note: You want to quantify all the amino acids.
5. In amino acid analysis, a protein sample hydrolyzed to individual amino acids is applied to a cation-exchange column. The amino acids are eluted by gradually increasing the pH of the mobile phase.
   a. Describe the principles of ion-exchange chromatography.
   b. Differentiate anion- versus cation-exchangers.
   c. Explain why changing the pH allows different amino acids to elute from the column at different times.

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Protein Quality Tests

Barbara A. Rasco

17.1 INTRODUCTION

Protein quality assays predict the nutritional quality of food proteins and how available these proteins are for growth and cell maintenance (1-3). Protein quality tests are designed to directly measure or estimate the dietary essential amino acid content of the test protein or protein-containing food, and how well the protein is digested, absorbed, and utilized for growth and maintenance.

Dietary amino acids are categorized as indispensable, conditionally indispensable, or dispensable, depending upon in vivo requirements for protein synthesis. Proteins with a higher ratio of indispensable amino acids have a higher biological value. Indispensable amino acids are: histidine, isoleucine, leucine, lysine, methionine, phenylanaline, threonine, tryptophan, valine; conditionally indispensable amino acids are: arginine, cysteine, tyrosine; and dispensable amino acids are: alanine, asparagine, aspartic acid, glutamine, glutamic acid, glycine, proline, and serine. In addition, the following amino acids may be indispensable under certain circumstances: taurine for infants; cysteine for preterm infants, older children with metabolic disorders, and malnourished patients with compromised liver function (e.g., cirrhosis); tyrosine for premature infants, the malnourished, or the elderly; and arginine, citrulline, and ornithine for the urea cycle (4).

Both biological (in vivo) and chemical or biochemical (in vitro) assays are used to predict protein quality. In vivo assays measure animal growth or nitrogen balance and predict how well a protein is metabolized and utilized by the body. In some cases, microbiological tests can be used to predict protein quality. In vitro assays are more commonly used than in vivo assays because they are quicker and less expensive than animal growth studies. Animal assays can take a month or more to conduct. In vitro assays for protein quality include enzyme assays that model mammalian digestion. Other in vitro tests compare the amino acid compositional data for a test protein and compare it with that of one or more reference proteins. This amino acid score is corrected for digestibility using either an in vivo or in vitro method to estimate protein quality. Chemical assays are used to predict the bioavailability of essential amino acids in food proteins.

17.2 GENERAL CONSIDERATIONS

17.2.1 Estimating Protein Requirements

Human protein requirements and recommended protein intake levels have been established by the Food and Agricultural Organization/World Health Organization (FAO/WHO) and the Food and Nutrition Board of the National Academy of Sciences (FNB/NAS). In 1985, FAO/WHO set the recommended protein intake level for adult humans at 0.75 g of protein/kg body weight per day, or 52.5 g of protein daily for a 70-kg adult based upon consumption of a protein with the digestibility of either milk or eggs (5). Recommended levels for infants and children are higher. Recommended protein intake levels are progressively lowered as children approach adulthood.

The FAO/WHO and the National Research Council (NRC) also have guidelines for essential amino acids (see Table 17-1 for FAO/WHO values). The protein quality tests outlined in this chapter can be used to determine how well a food or food protein ingredient meets these requirements.

17.2.2 Regulatory Actions and Protein Quality Tests

Selection and approval of protein quality methods are important from a regulatory perspective to meet public health needs and to protect consumers from economic fraud (12). Human clinical studies measuring growth or other metabolic indicators, such as nitrogen balance, provide the most accurate assessment of protein quality. However, clinical trials are usually inappropriate and clearly impractical for routine protein quality measurements, leaving animal assays as the most viable alternative.

One of the first widely used methods for assessing protein quality was the protein efficiency ratio (PER) developed in 1919 (described in section 17.3.1.1). PER measures the ability of test protein (relative to casein) to support the growth of young, rapidly growing rats. It has been used widely to predict protein quality for humans, and until recently, was the only method specified by the FDA for nutritional labeling purposes. However, PER overestimates the value of some animal proteins for human nutrition and underestimates the nutritive value of some proteins, particularly vegetable proteins (12-14). The PER method is time consuming and is criticized because it does not account for the value of a protein for maintenance. From 1981 through 1989 the Codex Committee on Vegetable Proteins evaluated procedures for assessing the nutritional quality of vegetable proteins for human nutrition. This evaluation eventually led to a recommendation by the Joint FAO/WHO Expert Consultation on Protein Quality Evaluation that a protein digestibility-corrected amino acid score (PDCAAS) method (described in section 17.3.4.2) be adopted internationally as the official method for routine evaluation of protein quality for humans instead of the PER assay. The PDCAAS method requires an accurate measurement of amino acid composition and a careful assessment of protein digestibility.

In 1990, the FDA proposed continued use of PER for protein quality assessment as part of the Nutritional Labeling and Education Act (NLEA). However, arguments raised by Young and Pellett [13], as well as comments received from the community at large as part of the informal rulemaking process, caused the FDA to reconsider their proposal and adopt the PDCAAS method. In 1991, the FDA adopted PDCAAS as an appropriate method for nutrition labeling purposes for all foods other than those intended for infants [14]. Details of the regulations for protein quality evaluation methods under NLEA are published in the *Federal Register* [15].

17.3 METHODS

17.3.1 Growth and Nitrogen Balance Techniques

Protein quality has traditionally been based largely on rat nutrition studies measuring nitrogen (N) balance or growth. Of the growth methods, the PER test has been used most widely. An improvement upon the PER method is the net protein ratio (NPR). For N balance, the biological value (BV) method and the net protein utilization (NPU) method are used. NPU is a modification of the BV method.

17.3.1.1 Protein Efficiency Ratio

The PER is a biological assay approved by AOAC International (AOAC Method 960.48)(16) to estimate protein quality of different foods or food ingredients. For this and other assays described below, details of the procedures can be found in the methods cited.

17.3.1.1.1 Principle The PER method is based upon the weight gain of a group of male weanling rats fed a test protein, compared to those fed a casein control diet in which casein is the sole source of dietary protein. The better the nutritional quality of the protein, the more rapidly the animals grow. The quality of the test protein is reported relative to the casein control. In general, a protein with a PER of >2.0 is of high quality, 1.5–2.0 is of intermediate quality, and <1.5 is of poor quality [4].

Since PER is an in vivo test, protein digestibility and amino acid bioavailability are encompassed to some extent within the scope of the assay. However, it is difficult from a PER assay to determine the individual contribution of each of these factors on protein quality.

17.3.1.1.2 Procedure Groups of male weanling rats from the same colony (21–28 days old) are fed 10% protein diets. There should be at least 10 animals per assay

group. One set of animals is fed a reference casein diet (control diet), and the other group(s) a diet containing the test protein(s). More than one test protein can be evaluated in the same experiment (multiple test groups). Any test protein must contain at least 1.80% nitrogen if it is to be incorporated in the test diet at the proper level by weight. Diets are isocaloric (same caloric content) and contain: carbohydrate in the form of corn starch, crude lipid as cottonseed oil, crude fiber as cellulose, and a balanced mix of vitamins and minerals. To account for differences in the protein content of different test materials, the amount of corn starch in the diet can be adjusted. Animals are housed in individual cages and are provided with appropriate assay diet and water ad libitum.

The weight of each animal is recorded at the beginning of the assay, and body weight and food intake are measured at regular intervals (at least every 7 days) during the 28-day feeding trial. PER is calculated as the weight gain per gram of protein (%N × 6.25) fed. PER is calculated using the average weight gain and average protein intake per each diet group (at day 28):

$$PER = \frac{weight\ gain\ of\ a\ test\ group\ (g)}{total\ protein\ consumed\ (g)} \quad [1]$$

An adjusted or corrected PER compares the quality of the test protein to reference casein. Casein is assigned a PER of 2.5, and results of the test protein are normalized to the value for casein in an attempt to reduce the interlaboratory variation that has been observed in collaborative experiments.

$$Adjusted\ or\ corrected\ PER =$$
$$\frac{PER\ of\ test\ protein}{PER\ of\ casein\ control} \quad [2]$$

17.3.1.1.3 Applications PER can discriminate between proteins based upon their nutritional quality, even though the test has a tendency to overestimate the protein quality of certain animal protein sources for the human diet and underestimate the value of some vegetable protein sources, as mentioned previously. The protein quality of vegetable proteins is underestimated because of the relatively higher need of the rapidly growing weanling rat for certain dietary essential amino acids compared to humans. From a public health standpoint, underestimating PER is not necessarily detrimental. However, there is a tendency of the PER assay to overestimate nutritional requirements for histidine, isoleucine, threonine, valine, and sulfur-containing amino acids (methionine and cystine). Also, casein is a less than ideal reference protein and is deficient by 15–30% in meeting the sulfur amino acid requirement of the rat (Table 17-1).

The major flaw with the PER method is that it is a growth assay, and as such, does not adequately account for the protein used for cell maintenance. A

17-1
table

Comparison of Suggested Patterns of Amino Acid Requirements for Humans with that of the Rat and with the Composition of Casein

Amino Acid	SUGGESTED PATTERN OF REQUIREMENT[1] (mg/g crude protein)					
	Infant Mean (range)[2]	Preschool Child (2–5 years)[3]	School Age Child (10–12 years)	Adult	Laboratory Rat[4]	Reported Composition Casein[5]
Histidine	26 (18–36)	(19)[6]	(19)	16	25	32
Isoleucine	46 (41–53)	28	28	13	42	54
Leucine	93 (83–107)	66	44	19	62	95
Lysine	66 (53–76)	58	44	16	58	85
Methionine + cystine	42 (29–60)	25	22	17	50[7]	35
Phenylalanine + tyrosine	72 (68–118)	63	22	19	66	111
Threonine	43 (40–45)	34	28	9	42	42
Tryptophan	17 (16–17)	11	(9)	5	12.5	14
Valine	55 (44–77)	35	25	13	50	63
Total						
Including histidine	460 (408–588)	339	241	127	407.5	531
Minus histidine	434 (390–552)	320	222	111	382.5	499

Adapted from (11) with permission.
[1]Values for humans from FAO/WHO/UNU (5).
[2]Amino acid composition of human milk (6–8).
[3]Amino acid requirement/kg divided by safe level of reference protein/kg. For adults, "safe" is taken as 0.75 g/kg; for children (10–12 years), 0.99 g/kg; for children (2–5 years), 1.10 g/kg. (This age range is chosen because it coincides with the age range of the subjects from whom the amino acid data were derived. The pattern of amino acid requirements of children between 1 and 2 years of age may be determined by taking an intermediate value between that of infants and preschool children.
[4]National Research Council (9), based on a protein requirement of 12% plus an ideal protein (100% true digestibility and 100% biological value).
[5]Steinke et al., 1980 (10).
[6]Values in parentheses interpolated from smoothed curves for amino acid requirements versus age.
[7]A lower requirement for the rat of 40 mg/g protein for methionine and cysteine has been reported (11).

protein that does not support growth has a PER of zero, even though it may be suitable for meeting protein requirements of adults. Problems such as this one have led to the recommendations to replace PER with other methods.

17.3.2 Net Protein Ratio

The net protein ratio (NPR) method is an animal growth assay that predicts the value of a test protein for cell maintenance. A protein may have a sufficient ratio of indispensable amino acids for cell maintenance even though the ratio is not high enough to support growth. The NPR method often is run in conjunction with PER. One group of animals is fed a nonprotein basal diet and a second group is fed a test diet. The average weight loss of the animals fed the nonprotein basal diet is recorded at 10 and 14 days. The NPR value calculated accounts for protein requirements for maintenance and represents the weight gain for animals on the test diet plus the average weight loss of animals on the zero protein diet per grams of protein consumed.

$$NPR = \frac{\text{weight gain of test animals (g)} + \text{weight loss of animals fed nonprotein basal diet (g)}}{\text{weight of protein consumed by test animals (g)}} \quad [3]$$

17.3.3 Biological Value and Net Protein Utilization

Unlike PER and NPR which are growth assays, biological value (BV) and net protein utilization (NPU) are determined from a nitrogen (N) balance assay. Nitrogen balance is calculated by measuring N intake by animals fed a test diet and subtracting metabolic and fecal N loss.

Nitrogen balance (B) = N intake
$$- (\text{N in feces} + \text{N in urine}) \quad [4]$$

The BV for a test protein is the proportion of absorbed nitrogen retained for maintenance or growth, corrected for metabolic and endogeneous losses of nitrogen:

$$BV = 100 \ (B - B_0)/A \quad [5]$$

where:

B = nitrogen balance (see Equation [4])
B_0 = N balance for animals fed a nonprotein diet
A = true nitrogen absorption:

A = N intake – (N in feces of animals fed test diet
$- $ N in feces of animals fed nonprotein diet) $\quad [6]$

or

$$BV = \frac{\text{ingested N} - (\text{total fecal N} - \text{endogenous fecal N})}{- (\text{total urinary N} - \text{endogenous urinary N})}}{\text{ingested N} - (\text{total fecal N} - \text{endogenous fecal N})}$$

[7]

The NPU is the *proportion* of nitrogen intake that is retained. NPU can be determined by comparing the carcass nitrogen content of a group of animals fed the test protein diet to that of a group of animals fed a zero protein diet.

$$NPU = \frac{\text{N retained}}{\text{N intake}} \times 100 = BV \times \text{true digestibility}$$ [8]

$$NPU = \frac{\begin{array}{c}\text{body N of test group}\\ -\text{ body N of nonprotein group}\\ +\text{ N consumed by nonprotein group}\end{array}}{\text{N consumed by test group}} \times 100$$ [9]

The biggest drawback of the nitrogen balance methods, specifically NPU, is that they fail to account for the differential rate of utilization or reutilization of dietary essential amino acids. A protein deficient in essential amino acids should have an NPU of zero. The rat has a mechanism of salvaging and reutilizing endogenous lysine so lysine-deficient proteins have NPU values of 30–50.

17.3.4 Amino Acid Scoring Patterns

17.3.4.1 Overview

Several protein quality testing methods utilize amino acid content data. Amino acid content of a test protein is compared to that of a reference protein. The estimate of protein quality is based upon either the first limiting amino acid or all of the essential amino acids. The protein quality estimate may be corrected for digestibility as determined by either an in vivo or an in vitro assay. Some methods that utilize amino acid content data make suitable quality control tests. When the amino acid scoring method incorporates a correction for protein digestibility, it may provide a more accurate assessment of the nutritional quality of a protein than PER.

Assays utilizing amino acid content data compare the amino acid composition of the test protein with that of egg, human milk, or bovine milk proteins, or with a reference pattern based upon human amino acid requirements. Because the amino acid requirements for growth and maintenance vary as people age, different reference patterns are used to estimate the nutritional quality of a given protein for infants, older children, and adults (Table 17-1). For the amino acid scoring method described below (section 17.3.4.2), the reference pattern for preschool age children is recommended for evaluating protein quality for all groups

except infants, even though this may overestimate protein requirements and underestimate protein quality for adults and older children . For infants, the recommended scoring pattern is the amino acid composition of human milk.

The protein quality evaluation methods based upon amino acid data require accurate amino acid analysis of a food protein or a food product. The amino acid composition generally is determined by hydrolyzing the protein to its constituent amino acids and then separating the amino acids chromatographically (see Chapter 16). Separate assays are required for cystine, methionine, and tryptophan.

17.3.4.2 Protein Digestibility—Corrected Amino Acid Score (PDCAAS) Method

17.3.4.2.1 Procedures To calculate the amino acid score for each dietary essential amino acid, divide the amount of each essential amino acid in a given test protein by the amount in the reference protein. According to the PDCAAS procedure, the reference protein used to calculate the amino acid score is actually the reference pattern of the 1985 FAO/WHO requirements for 2- to 5-year-olds (see Table 17-1). The amino acid with the lowest ratio is the first limiting amino acid. The value also is the amino acid score for the test protein. The amino acid score is expressed either as a ratio compared to the standard, or as a percentage.

$$\text{Amino acid score (uncorrected)} = \frac{\text{mg of amino acid in 1 g of test protein}}{\text{mg of amino acid in 1 g of reference protein}}$$ [10]

The amino acid score is equivalent in practice to chemical score and protein score methods. The chemical score was originally defined relative to the amino acid composition of egg protein. The amino acid score combined with a determination of protein digestibility (see section 17.3.7) is the basis of the protein digestibility-corrected amino acid score (PDCAAS) method.

PDCAAS = amino acid score for limiting amino acid
         × % true digestibility   [11]

17.3.4.2.2 Applications Unless the amino acid score is corrected for digestibility, it may not be a good reflection of protein quality. An amino acid score may not be valid for some mixtures of food proteins, even though a value could be readily calculated knowing the amino acid composition of each protein in the composite and the relative amounts of each. Likewise, a calculated value of protein quality for various foods may not provide a good indication of the overall protein quality of a diet containing a wide variety of foods. This is

because the body's utilization of dietary protein is affected by a number of different factors that are not reflected in an amino acid score. This includes the presence of antinutritional factors such as enzyme inhibitors that could impact how a protein is digested and absorbed. Also, the methods do not differentiate between D and L forms of amino acids.

There is a general consensus that the PDCAAS method can provide a better estimate of protein quality for humans than the PER rat growth assay. The PDCAAS method is recommended by the FAO/WHO for measuring protein quality (12) and has been adopted by the FDA in regulations promulgated under the NLEA (see section 17.2.2) for measuring protein quality of all foods except those intended for infants (15).

17.3.5 Calculated PER and Discriminate Calculated PER

17.3.5.1 Procedures

Amino acid composition data for all essential amino acids in a food are compared to the FAO/WHO standard when calculating the calculated PER (C-PER) (AOAC Method 982.30). The C-PER is a PER calculated from the amino acid composition of the test protein and an in vitro protein digestibility measurement (see section 17.3.7). A related method for estimating protein quality, the discriminate calculated PER (DC-PER) (AOAC Method 982.301), is calculated using only the essential amino acid composition of the food compared to the FAO/WHO standard. The calculations for both C-PER and DC-PER involve complicated algorithms that are provided as part of the AOAC procedures.

17.3.5.2 Applications

Unlike the amino acid score, C-PER and DC-PER take into consideration the content of all of the dietary essential amino acids. This consideration is useful, especially in foods in which more than one essential amino acid is present in relatively low amounts.

The C-PER and DC-PER methods are intended to be alternate methods for routine screening of foods or protein ingredients for protein quality. Used together, they can provide a reliable estimate of protein quality for a majority of foods and food ingredients. Most of the concerns about the C-PER method relate to the reliability of the in vitro digestibility measurements (see section 17.3.7).

17.3.6 Essential Amino Acid Index

17.3.6.1 Procedure

The essential amino acid index is calculated by taking the ratio of the test protein to the reference protein for each of the eight essential amino acids plus histidine using this equation:

$$\text{Essential amino acid index} =$$

$$\sqrt[9]{\frac{\text{mg of lysine in 1 g of test protein}}{\text{mg of lysine in 1 g of reference protein}} \times \begin{array}{c}\text{etc. for all 8} \\ \text{essential amino} \\ \text{acids} \\ \text{+ histidine}\end{array}} \quad [12]$$

Methionine and cystine are counted as a single amino acid in Equation [12], as are phenylalanine and tyrosine (see practice problem 3 in section 17.6).

17.3.6.2 Applications

The essential amino acid index method is a rapid method for evaluating food formulations for protein quality. Like the C-PER and DC-PER methods, it is calculated using the content of all dietary essential amino acids. However, unlike the PDCAAS and C-PER methods, it does not include an estimate of protein digestibility. Therefore, this index would not account for differences in protein quality due to the effect of various processing methods or certain chemical reactions (e.g., browning reactions) that can adversely impact how a protein is digested.

17.3.7 Protein Digestibility Assays

All proteins are digested, absorbed, and utilized by our bodies to different extents. Differences in protein digestibility arise from the susceptibility of a protein to enzymatic hydrolysis in the digestive system. This is directly related to the primary, secondary, and tertiary structure of the protein. The presence of nonprotein dietary constituents consumed at the same time as the protein also can affect protein digestion. Some of these components include phytate, dietary fiber, and various toxigenic agents that inhibit proteolytic enzymes. How a protein has been treated or processed also is important. Processing and storage conditions can alter the three-dimensional structure of the protein. Processing may increase a protein's susceptibility to digestive enzymes because more peptide linkages are exposed. However, chemical changes also may occur that reduce a protein's susceptibility to digestive enzymes. In addition, chemical reactions that involve amino acids, such as Maillard browning, can significantly reduce the biological availability of dietary essential amino acids, particularly lysine.

17.3.7.1 In Vivo Assays

Protein digestibility measures the proportion of protein nitrogen absorbed. Common in vivo digestibility

assays provide the best indication of protein digestibility in humans by measuring nitrogen balance in an animal assay.

17.3.7.1.1 Procedures (True Protein Digestibility: AOAC Method 99.629) Male weanling rats (50–70 g) are initially fed a protein-free diet for a 4-day preliminary period and then for a 5-day balance period (total 9 days). On each day of the 5-day balance period, the weight of the feed consumed is determined in addition to the weight of any spilled food. The feces also are collected during the 5-day balance period and weighed. Separate groups of rats are fed either a test diet (10% protein) or a nonprotein diet concurrently. Diets are fed at a rate of 15 g (dry matter)/day. The nitrogen content of the feces is determined by the Kjeldahl method (AOAC Method 955.04C, 976.05; see also Chapter 15). Test diets are analyzed for protein nitrogen, moisture (AOAC Method 927.05 or 934.01), fat (AOAC Method 920.38A, 983.23 or an equivalent method), and dietary fiber (AOAC Method 985.29).

The true digestibility is calculated based upon the amount of nitrogen ingested and feed intake, corrected for metabolic losses in the feces.

$$\text{True digestibility (\%)} = \frac{Ni - (Fn - Mn)}{Ni} \times 100 \quad [13]$$

where:

Ni = N intake
Fn = fecal N (test protein group)
Mn = fecal metabolic N loss (nonprotein group)

If the correction for metabolic losses in the feces is not made, the value is termed apparent digestibility:

$$\text{Apparent digestibility (\%)} = \frac{Ni - Fn}{Ni} \times 100 \quad [14]$$

17.3.7.1.2 Applications Protein digestibility data from rat studies must be used with caution when estimating protein quality for humans. When at all possible, protein quality evaluation should be obtained from nitrogen balance studies with humans. Obviously, this is not always possible due to safety, ethical, or monetary restraints. Fortunately, where there are comparative data available from nitrogen balance studies for the same food proteins, the results from rat and human studies are similar (12).

17.3.7.2 In Vitro Assays

17.3.7.2.1 Overview Various in vitro enzymatic hydrolysis methods have been proposed to evaluate the digestibility and availability of proteins, usually by a one- or two-step process using mammalian gastric, pancreatic, or intestinal enzymes or other proteolytic

enzymes including those from bacterial sources (see C-PER method in section 17.3.7.2.2). Some of the enzymes or combinations of enzymes that have been used include: (1) pepsin, (2) pepsin–pancreatin, (3) papain, (4) papain–trypsin, (5) trypsin, (6) trypsin–chymotrypsin–peptidase, and (7) trypsin–chymotrypsin–peptidase–bacterial protease (Pronase P or E).

In vitro digestibility assays can be classified as methods that measure either the extent of hydrolysis or the initial rate of protein hydrolysis. Further classifications are based upon the enzyme used and the method of digest fractionation (if used). In the in vitro digestibility assays, the amount of protein-containing ingredient or food (based upon protein nitrogen content), pH, and temperature of the incubation medium generally are fixed based on the requirements of the enzyme reactions(s). The enzyme-to-substrate ratio often varies depending on the enzymes used. The enzyme-to-substrate ratio can influence reaction rate, the type and size of the peptides generated during hydrolysis, and the enzymatic release of different amino acids. Since the accumulation of digestion products during proteolysis may inhibit the enzyme reaction, some methods include procedures to remove these digestion products from the reaction mixture. Protein digestibility can be determined directly from the enzymatic hydrolysate or after the hydrolysate has been further treated.

Values from in vitro digestibility assays generally do not take into account fermentation of food proteins in the lower bowel or the amino acid balance of the protein tested. In vivo tests, such as rat nitrogen balance or amino acid balance, generally provide a better assessment of protein digestibility for processed or complex food mixtures than an in vitro assay. This is because the susceptibility of proteins to enzymatic hydrolysis may be altered as a result of processing. Also, other food components besides proteins in a complex food mixture may interfere with enzymatic hydrolysis.

17.3.7.2.2 pH-Shift Method An in vitro digestibility assay is conducted as part of the C-PER assay as described previously to correct an estimate of protein quality based on amino acid composition for the digestibility of the protein (see sections 17.3.5 and 17.3.6). The degree of digestibility of a test protein is calculated relative to casein. The assays are based upon a drop in pH that occurs as a protein is hydrolyzed. Proteases break peptide bonds, releasing carboxyl groups and liberating H⁺ ions which causes the pH of the reaction mixture to drop. This is the reason these methods are sometimes called pH-shift or pH-drop procedures.

AOAC Method 982.30 for in vitro digestibility is based upon studies by Hsu et al. (17) and Satterlee et al. (18) and are summarized in Fig. 17-1. The "digestibility" component of the C-PER assay is conducted

C-PER METHOD

Put specified quantity of protein (test protein or casein) (based on Kjeldahl N) in solution at 37°C and adjust to pH 8.0.

↓

Add freshly prepared trypsin, chymotrypsin, and peptidase solutions (type, activity and amounts of these enzymes are specified as are preparation procedures).

↓

After 10 min, add bacterial protease (specified amount and activity).

↓

Transfer to 55°C water bath.

↓

Transfer back to 37°C bath at 19 min.

↓

Read pH at 20 min.

↓

Calculate digestibility as:

% Digestibility = 234.84 − 22.56(x)

x = pH at 20 min.

17-1
figure

Procedure for in vitro digestibility assay for C-PER assay (AOAC Method 982.30).

rapidly and is unaffected by food lipids and buffering salts commonly found in foods. This digestibility assay is sensitive enough to detect the presence of soybean trypsin inhibitors and to detect changes in protein digestibility that may occur during processing. However, there are problems with digestibility assays that are part of the C-PER method for certain foods, including those with high levels of connective tissue proteins, and with low protein foodstuffs in general. The C-PER and DC-PER values may not be similar and may not correlate well with biological methods for assessing protein quality for foods that contain large amounts of cell wall materials (e.g., yeast, bran), partially digested proteins, or protease inhibitors.

Although in vitro digestibility values for pH-shift methods generally correlate well with data for in vivo digestibility assays when ranking protein sources, they do not accurately estimate quantitative differences between samples with low and high protein digestibility. The major limitation of the pH-shift method is that the pH is not constant during the course of the reaction. The buffering capacity of peptides, proteins, and other substances in the food may influence how the pH changes during this type of assay and not reflect the true digestibility of the protein (19).

17.3.7.2.3 pH-Stat Method To overcome the problems with pH-shift assays, Pedersen and Eggum (20) developed a protein digestibility assay in which the pH of the reaction mixture was kept constant during the incubation period (Fig. 17-2). This is the pH-stat method. The same enzymes are used as in the pH-shift method (summarized in Fig. 17-1). Protein digestibility in the pH-stat method is estimated from the volume of a standard base (0.1 N NaOH) added during the incu-

pH-STAT METHOD

Put specified quantity of protein (test protein or casein) (based on Kjeldahl N) in solution at 37°C and adjust to pH 8.0.

↓

Add suspension of trypsin, chymotrypsin, and peptidase (type, activity, and amounts of these are specified in assay procedure)

↓

Record the amount of 0.1 N NaOH required to maintain pH at 7.98 for exactly 10 min.

↓

Calculate true digestibility as:

% True digestibility = 76.14 + 47.77B

B = ml of 0.1 N NaOH added

17-2
figure

Procedures for pH-stat method for in vitro digestibility. [Adapted from (20).]

bation to maintain a constant pH of 8.0 during the enzyme incubation. The pH-stat assay is generally more accurate than a pH-shift assay and provides a better correlation with in vivo digestibility values. Pedersen and Eggum (20) used a single equation to calculate in vitro digestibility by the pH-stat method for 31 plant and animal proteins and obtained a correlation coefficient of >0.90 with in vivo digestibilities for the same proteins.

Other researchers have sought to improve upon the pH-stat procedure developed by Pedersen and Eggum (20), in particular the equation to calculate digestibility. For example, Dimes and Haard (21) used a pH-stat procedure to measure the degree of protein hydrolysis as enzymatic digestion proceeds.

17.3.7.2.4 Immobilized Enzyme Assay In a recently developed in vitro digestibility assay, digestive tract enzymes are covalently immobilized on large-pore-diameter (2000 Å) glass beads via an amide linkage (22). The protein sample is passed through a biodigester that contains immobilized pepsin and a second biodigester containing immobilized trypsin, chymotrypsin, and intestinal mucosal peptidases. Primary amines in the digested sample react with o-phthalaldehyde (OPA) and the fraction of total peptide bonds hydrolyzed is calculated from the absorbance values. The method is time consuming but has a number of advantages and results correlate well with rat bioassays for a wide variety of food proteins.

17.3.8 Amino Acid Availability

17.3.8.1 Overview

The amino acid availability method for protein measures the relative digestibilities of the individual amino acids. Amino acids in proteins may be digested and absorbed at different rates, for various reasons, and affect protein utilization. For example, the rate of amino acid uptake from a protein mixture compared

with a protein supplemented with free amino acids differs even if the amino acid composition is the same for both. Free amino acids are absorbed more quickly than amino acids in protein.

Amino acid scoring pattern methods are based on the assumption that there is a direct linear relationship between the concentration of a limiting amino acid and utilization of the limiting amino acid in a protein. A second assumption is that the amino acid balance in a protein has no effect on the utilization of dietary essential amino acids, particularly the limiting amino acid. In addition, the amino acid balance plays an important role in the overall quality of food proteins, but this is not generally reflected in the amino acid scoring pattern assay corrected for digestibility, particularly if an in vitro method for protein digestibility was used.

Because an amino acid analysis is conducted on a protein that has been acid hydrolyzed, it often does not provide a good indication of the protein bioavailability. A number of factors can affect how a protein is digested. For example, if the secondary or tertiary structure of a protein has been altered during heating, or by some other processing treatment, protein digestibility may increase because peptide bonds become more accessible. However, common reactions in foods such as Maillard browning reactions that cause covalent modification of amino acids generally lower protein digestibility.

Also, the amino acid composition does not provide an indication of how well an amino acid will be utilized, so other assays are needed to determine availability of individual essential amino acids. For example, foods that undergo Maillard browning are particularly susceptible to loss of lysine. Sulfur-containing amino acids (methionine and cysteine) also can be lost during processing. Moderate to severe heating that causes proteins to aggregate results in lower protein digestibility. Severe heat treatment can damage protein to such a degree that digestibility is reduced and bioavailability of all dietary essential amino acids is affected.

17.3.8.2 In Vivo Amino Acid Availability

Conducting an in vivo assay for amino acid availability is similar to conducting an assay for apparent protein digestibility. However, instead of simply measuring the nitrogen content of the diet and the feces, the amino acid profile of each is determined. The amino acid balance can be calculated for all amino acids, but generally in practice it is restricted to the first, or first and second, limiting amino acids:

$$Amino\ acid\ balance =$$
$$amino\ acid\ intake\ (g)$$
$$- amino\ acid\ excreted\ (fecal\ content)\ (g)\qquad [15]$$

In vivo amino acid digestibility can overestimate

protein quality because a significant fraction of certain limiting dietary essential amino acids (lysine, methionine, cystine, threonine, and tryptophan) are lost through microbial fermentation in the large intestine.

17.3.8.3 Microbiological Assays for Amino Acid Availability [AOAC Method 960.47]

Microbiological assays using the bacteria *Streptococcus zymogenes* or *Pediococcus cerevisiae (acidilacti)*, or the protozoan *Tetrahymena pyriformis W* can be used to estimate amino acid availability. The test protein first is treated with a proteolytic enzyme or enzyme preparation (e.g., papain) prior to introduction of microbes into the incubation mixture to reduce the time needed for the assay. The microbe is incubated in media containing the partially hydrolyzed test protein. Several concentrations of the test protein are used. Microbes are enumerated at the end of the incubation period, which can be several days. Microbial growth is proportional to the level and bioavailability of the amino acid.

S. zymogenes can be used to assay available arginine, histidine, leucine, isoleucine, valine, methionine, and tryptophan; the organism does not require lysine and cannot be used to assay for this amino acid. Microbial assays for lysine often use the organism *P. cerevisiae*. The protozoan *T. pyriformis* assay can be used to assay for the following amino acids: arginine (required for the rat), histidine, isoleucine, leucine, lysine, methionine + cystine, phenylalanine + tyrosine, threonine, tryptophan, or valine. Data from the *T. pyriformis* assay correlate well with those from rat bioassays. However, several common food additives including propionates, benzoates, sorbates, nitrate, erythorbate, ascorbate, and certain spices interfere with the protozoan assay.

17.3.8.4 In Vitro Amino Acid Availability

Amino acid availability can be measured by in vitro enzymatic digestion utilizing assay systems that mimic mammalian digestion. Similar to in vitro tests for protein digestibility, these assays are useful for ranking proteins, particularly when the effect of processing treatments on a given type of food proteins is being evaluated. One common in vitro amino acid availability test measures the amino acid composition of a filtrate recovered after a test protein has been hydrolyzed with a mixture of trypsin, pepsin, and pancreatin.

17.3.9 Availability of Essential Amino Acids

17.3.9.1 Overview

The free amino group on the side chain of lysine can react with many constituents during food processing (heating, drying, etc.) and storage to produce biologically unavailable lysine complexes. These lysine com-

basic amino groups of lysine, histidine, or arginine often correlates well with bioassays. These assays are relatively rapid and are particularly useful for monitoring heat damage to oilseed and cereal proteins. Results are less reliable for fish and meat proteins. Azo dyes can bind to the basic reaction products formed early in Maillard browning and because of this, cannot be used to detect heat damage to milk proteins. For dried milk and other susceptible products, a dye-binding assay utilizing Remazol Brilliant Blue R can be helpful for detecting the initial products of Maillard browning reactions. Remazol Brilliant Blue R reacts with free amino acid groups of lysine and also the thiol group of cystine.

17.3.9.3 Assays for Sulfur-Containing Amino Acids

The sulfur-containing amino acids, methionine and cysteine/cystine, are often the limiting amino acids in foods. Because these amino acids can be readily oxidized to nonbioavailable forms during drying, bleaching, and other processing operations, having suitable assay methods for nutritionally available forms is important.

Available cystine/cystine can be measured by converting cystine to cysteine with dithiothreitol, reacting cysteine with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and measuring the quantity of the derivatized form.

Methionine can reduce dimethyl sulfoxide (Me_2SO) to dimethyl sulfide (Me_2S), which can be quantified by headspace gas chromatography. Results from the dimethyl sulfide method correlate well with results from biological assays for methionine. Methionine reacts with cyanogen bromide (CNBr), producing methylthiocyanate (MeSCN), which can be measured by gas chromatography and is also a good indication of available methionine. The oxidized forms of methionine, methionine sulfone and methionine sulfoxide, are not measured by either of these methods.

17.4 SUMMARY

Biological assays, principally rat feeding studies, have been commonly used to predict protein quality for humans by measuring either nitrogen balance (BV and NPU methods) or growth (PER or NPR methods). The PER method involves feeding a set of male weanling rats a test diet containing a single source of dietary protein at a fixed level for a set period, monitoring food intake and weight gain. The PER procedure with casein as the reference protein was the protein quality test method specified by the FDA for nutritional labeling purposes until recently. However, problems associated with the PER method and improvements in chromato-

graphic methods for amino acid composition have led to the adoption of the PDCAAS method for most nutrition labeling purposes.

The PDCAAS method involves calculating the amino acid score and determining protein digestibility for the test protein. Other methods utilize amino acid composition data with (C-PER) or without [(DC-PER), essential amino acid index)] a correction for protein digestibility. While the PDCAAS method uses an in vivo (rats) determination of protein digestibility, the C-PER procedure uses an in vitro assay that measures a pH drop resulting from enzymatic digestion of the test protein. Improvements in this in vitro assay for digestibility include the pH-stat method and the immobilized digestive enzyme assay.

Bioavailability of essential amino acids can be tested by in vivo and in vitro methods including microbiological assays. Chemical or microbiological tests predict the availability of limiting amino acids as nutrients, particularly lysine and the sulfur-containing amino acids. These amino acid bioavailability assays are helpful for evaluating the effects of various processing treatments on the limiting amino acids in a given food without having to resort to more extensive animal experiments.

17.5 STUDY QUESTIONS

1. Provide the amount of protein per serving, expressed as a percent of the Daily Value, on a nutritional label for a new food product (not a food intended for infants).
   a. What method must be used to estimate protein quality?
   b. Outline the procedure for the protein quality assay in (1a) and calculations required.
2. Describe an animal assay that can predict the adequacy of a food protein for growth and maintenance. Discuss two limitations of the method you choose.
3. Define and briefly describe the differences between the following assay procedures:
   a. PER versus Adjusted PER
   b. PER versus NPR
   c. PER versus BV
   d. BV versus NPU
   e. amino acid score versus essential amino acid index
   f. PER versus C-PER
   g. C-PER versus DC-PER
   h. PDCAAS versus amino acid score
   i. true digestibility versus apparent digestibility
   j. pH-shift versus pH-stat method for in vitro digestibility
4. How are certain microorganisms used to measure amino acid availability?
5. Explain how in vitro assays can be used to assess (a) protein digestibility and (b) amino acid availability. What are the advantages and disadvantages of an in vitro assay compared to an in vivo assay?
6. You are helping to develop a new process for making a high-protein snack food from cereal grains and soy. You

plexes are produced through reaction with reducing sugars (producing Maillard reaction products), oxidized polyphenols (including caffeic acid present in oilseeds), oxidized lipids, glutaminyl and asparatinyl residues (during severe roasting or heating operations), and alkaline solutions (destroy or racemize lysine, produce lysinoalanine). Bioavailable lysine is not equal to lysine content. Essentially, the only source of bioavailable lysine in a food is the lysine residue with a free ε-amino group (reactive lysine).

Reactive lysine is measured directly using 1-fluoro-2,4-dinitrobenzene (DNFB), trinitrobenzenesulfonic acid (TNBS), ω-methylisourea, or o-phthalaldehyde. Reactive lysine is measured indirectly by the DNFB difference method, dye-binding procedures, a fluorescence method, or reduction by $NaBH_4$. Several microbiological assays (see section 17.3.8.3) have been developed to measure amino acid bioavailability, as have in vitro enzymatic hydrolysis methods (see section 17.3.8.4). In vitro digestibility assays assume that protein digestibility provides good approximation of the digestibility of each amino acid, including reactive lysine. Enzymatic hydrolysis with pepsin plus pancreatin or with pronase makes use of the fact that these proteolytic enzymes release only reactive lysine. A comparison of different methods for bioavailable lysine is given in Table 17-2.

17.3.9.2 Assays for Lysine

17.3.9.2.1 Assays with 1-Fluoro-2,4-Dinitrobenzene
A test protein is reacted with 1-fluoro-2,4-dinitrobenzene (DNFB, also referred to as FDNB) (AOAC Method 975.44). DNFB reacts with the free ε-amino group in lysine. The amino acid profiles of the DNFB-treated test protein as well as the untreated test protein are determined. The amount of available lysine is the amount of lysine in the untreated protein minus that in the DNFB-treated sample.

Spectrophotometric assays for available lysine involve reaction of DNFB with protein, hydrolysis of the protein in acid, and comparison of the amount of

DNFB-reactive lysine (g/16 g of nitrogen) with a standard (mono-ε-N-dinitrophenyl lysine hydrochloride monohydrate [DNP-lysine]). The DNFB-reactive lysine generally provides a good indication of bioavailable lysine in oilseeds, milk powder, and fish flour. The method is less suitable for partially hydrolyzed proteins such as hydrolyzed vegetable and meat proteins and certain fish meals, or protein foods containing high concentrations of reducing sugars, including some cereals. Sugars released during acid hydrolysis can reduce up to 30% of the DNP-lysine derivatives so they are no longer measurable. Adding excess DNFB to the reaction mixture can have a protective effect when these foods are assayed.

17.3.9.2.2 Assays with Trinitrobenzenesulfonic Acid
The water-soluble reagent, trinitrobenzenesulfonic acid (TNBS) can be used for free lysine measurement. However, TNBS-lysine derivatives are more susceptible to loss during acid hydrolysis than DNFB derivatives. Like DNFB, TNBS reacts with lysine derivatives formed early in Maillard browning which may remain or may no longer be bioavailable. The TNBS derivatives formed during Maillard browning will break down to yield labeled lysine complexes, whereas DNP derivatives will not.

17.3.9.2.3 Enzymatic Methods
Enzymatic methods for bioavailable lysine are particularly useful for carbohydrate-containing foods. Lysine decarboxylase has a high degree of specificity for L-lysine, yielding carbon dioxide and the biogenic amine cadaverine, either of which can be easily measured by gas chromatography or other methods. Unfortunately, there is little comparative data between this method and bioassay procedures for amino acid availability. A comparison of results from different assays for available lysine is given in Table 17-2.

17.3.9.2.4 Dye-Binding Methods
The binding capacity of azo dyes such as Orange 12 (Acrilane Orange G, 1-phenylazo-2-naphthol-6-sulfonic acid) to the free

17-2 table   Lysine Levels (mg/g N) in Four Samples of Milk Powder

Sample of Milk Powder	Total Value after Acid Hydrolysis[1]	Lysine Reacting with DNFB[2]	Lysine Released by In Vitro Enzymatic Digestion	Value Obtained by Growth Assay with the Rat
Good quality	500	513	519	506
Slightly damaged	475	400	388	381
Scorched	425	238	281	250
Severely scorched	380	119	144	125

From (1), used with permission.
[1]By amino acid analysis.
[2]DNFB = 1-fluoro-2, 4-dinitrobenzene.

want to determine the protein quality of the snack food under various processing (toasting and drying) conditions. Considering the number of samples to be tested, you cannot afford an expensive in vivo assay, and you cannot wait more than a few days to get the results.

a. Briefly describe the procedure you would use to estimate and compare the protein quality of the snack food made under different processing conditions. Include an explanation of the principles involved.

b. You suspect that certain time-temperature combinations lead to overprocessed products. Your testing from (6a) shows that these samples have a lower nutritional quality. What amino acids(s) in the snack food would you suspect to be the most adversely affected by thermal abuse?

c. What test(s) could you use to confirm that amino acid(s) have become nutritionally unavailable by overprocessing? How are these tests conducted?

17.6 PRACTICE PROBLEMS

1. PER, RPER, NPR and apparent digestibility.
   Based on the information below, calculate the (a) PER, (b) RPER, (c) NPR, and (d) apparent digestibility for casein, ingredient X(Ing. X), and ingredient X with supplemental amino acids (AA).

Weight Gain and Food Intake for Animals Fed Protein Ingredient X, Protein Ingredient X with Supplemental Amino Acids, or Casein

	NONCUMULATIVE WEIGHT GAIN (g)			
	wk1	wk2	wk3	wk4
Casein	31.2	30.0	31.2	27.0
Ing. X + AA	26.1	28.7	24.3	30.6
Ing. X	3.0	4.8	6.4	4.9

	FOOD INTAKE (g)			
	wk1	wk2	wk3	wk4
Casein	90.3	134.5	112.1	147.9
Ing. X + AA	91.3	131.6	101.0	129.6
Ing. X	80.7	74.2	52.3	64.6

Casein was added at 12% of the diet by weight and ingredient X or ingredient X with supplemental amino acids at 24.7% of the diet by weight. The protein concentration of casein (%N × 6.25) is 83.3% and that of ingredient X, 40.4%.

In addition, a group of rats was fed a basal diet (no protein) in conjunction with the study described earlier. By day 14, these animals had lost an average of 14.8 g each. The following data were collected in a related study to determine apparent digestibility, with each diet containing 9.1% protein:

	Food Intake (g)	Fecal Nitrogen (g)
Casein	260	0.27
Ing. X + AA	231	0.40
Ing. X	117	0.27

2. Protein digestibility
   Calculate the in vitro digestibility for the following proteins using the pH-shift method (AOAC Method 982.30). For soy, the final pH was 6.7; for whey, the final pH was 6.3.

3. Essential amino acid index, amino acid score, and PDCAAS.
   Using the date provided in the table below,
   a. Calculate the essential amino acid index for defatted soy flour.
   b. Determine the amino acid score for the soy flour.
   c. Calculate the PDCAAS, using the true digestibility value of 87% for defatted soy flour (15).

Amino Acid	Soy[1] (mg/g protein)	Reference Pattern[2] (mg/g protein)
Isoleucine	46	28
Leucine	78	66
Lysine	64	58
Methionine/cystine	26	25
Phenylalanine/tyrosine	88	63
Threonine	39	34
Tryptophan	14	11
Valine	46	35
Histidine	26	19

[1]From (23).
[2]From (5).

4. Amino acid bioavailability.
   Determine by microbiological assay the level of tryptophan in a nutritional supplement containing free amino acids, but no protein ingredients. A stock culture of Lactobacillus plantarum (ATCC #8014) was used with L-tryptophan as a standard (working standard solution, 5 µg/ml). One (1.0) gram of the nutritional supplement (sample) was suspended in 50 ml of distilled water, heated in an autoclave for 10 min at 121-123°C, and filtered.

Volume of Working Standard Solution	Concentration of Working Standard Solution	% Transmission
0 ml	0 µg	95
1	5	83
2	10	76
3	15	70
4	20	67
5	25	63

Volume of Diluted Sample (ml)	% Transmission
0	90
0.2	80
0.5	69

What is the concentration of the tryptophan in the nutritional supplement?

Answers

1a. PER preliminary calculations:

Casein control group
  Total weight casein group = 120 g
  Total food intake casein group = 485 g
  Amount of protein consumed by casein group = 485 g ×
    12/100 × 83.3/100 = 48.5 g

Ing X + A:
  Wt gain = 110 g
  Food intake = 453 g
  Protein consumed = 45.2 g

Ing X:
  Wt gain = 19 g
  Food intake = 272 g
  Protein consumed = 27.1 g

PER calculations:
  PER = wt gain/protein intake
  Casein: 120/48.5 = 2.47
  Ing X + AA: 110/45.2 = 2.43
  Ing X: 19/27.1 = 0.70

1b. Calculate the RPER for Ing. X and Ing. X + AA:
  RPER = (PER of ingredients/PER of casein) × 2.5
  Ing X + AA: (2.43/2.47) × 2.5 = 2.45
  Ing. X: (0.70/2.47) × 2.5 = 0.71

1c. $\text{NPR} = \dfrac{\text{wt gain} - \text{wt loss (g) (zero protein)}}{\text{protein intake (g)}}$

  Casein: (120 − 14.8)/48.5 = 2.2
  Ing. X + AA: (110 − 14.8)/45.2 = 2.1
  Ing. X: (19 − 14.8)/27.1 = 0.15

1d. Apparent digestibility =

$$\frac{\text{g nitrogen ingested} - \text{g nitrogen in feces}}{\text{g nitrogen ingested}} \times 100$$

Percent nitrogen in diet, for example, for casein: 260 g diet
× (0.091/6.25) = 3.79 g of nitrogen
Apparent digestibility for casein: (3.79 − 0.27)/3.79 × 100 =
93%

For Ing. X + AA: (3.36 − 0.4)/3.36 × 100 = 88%
For Ing. X: (1.70 − 0.27)/1.70 × 100 = 84%

2. Percent protein digestibility (in vitro) = 238.84 − 22.56x
  For soy: 238.84 − 22.56(6.7) = 88%
  For whey: 238.84 − 22.56(6.3) = 97%

3a.

$$\sqrt[9]{\frac{(46/28)(78/66)(64/58)(26/25)}{(88/63)(39/34)(14/11)(46/35)(26/19)}} =$$

$\sqrt[9]{(1.64)(1.18)(1.10)(1.04)(1.39)(1.15)(1.27)(1.31)(1.37)} = 1.26$

3b. Amino acid score = 1.04

3c. PDCAAS = amino acid score × true digestibility = 1.04
  (0.87) = 0.905

4. Construct a standard curve % Transmission (y-axis) versus tryptophan (μg) (x-axis). From the standard curve,

estimate the concentration of the tryptophan in the two
dilutions:

  0.2 ml of diluted sample ⇒ ~1.5 μg of tryptophan
  0.5 ml of diluted sample ⇒ ~3.3 μg of tryptophan

Average is 7.0 μg/ml.
Concentration is 7.0 μg of tryptophan/ml × 50 ml/g of
supplement = 350 μg of tryptophan/g of supplement.

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Casein control group

Total weight casein group = 120 g

Total food intake casein group = 485 g

Amount of protein consumed by casein group = 485 g × 12/100 × 83.3/100 = 48.5 g

Ing X + A:

Wt gain = 110 g

Food intake = 453 g

Protein consumed = 45.2 g

Ing X:

Wt gain = 19 g

Food intake = 272 g

Protein consumed = 27.1 g

PER calculations:

PER = wt gain/protein intake

Casein: 120/48.5 = 2.47

Ing X + AA: 110/45.2 = 2.43

Ing X: 19/27.1 = 0.70

1b. Calculate the RPER for Ing. X and Ing. X + AA:

RPER = (PER of ingredients/PER of casein) × 2.5

Ing X + AA: (2.43/2.47) × 2.5 = 2.45

Ing. X: (0.70/2.47) × 2.5 = 0.71

1c. $NPR = \dfrac{\text{wt gain} - \text{wt loss (g) (zero protein)}}{\text{protein intake (g)}}$

Casein: (120 − 14.8)/48.5 = 2.2

Ing. X + AA: (110 − 14.8)/45.2 = 2.1

Ing. X: (19 − 14.8)/27.1 = 0.15

1d. Apparent digestibility =

$\dfrac{\text{g nitrogen ingested} - \text{g nitrogen in feces}}{\text{g nitrogen ingested}} \times 100$

Percent nitrogen in diet, for example, for casein: 260 g diet × (0.091/6.25) = 3.79 g of nitrogen

Apparent digestibility for casein: (3.79 − 0.27)/3.79 × 100 = 93%

For Ing. X + AA: (3.36 − 0.4)/3.36 × 100 = 88%

For Ing. X: (1.70 − 0.27)/1.70 × 100 = 84%

2. Percent protein digestibility (in vitro) = 238.84 − 22.56x

For soy: 238.84 − 22.56(6.7) = 88%

For whey: 238.84 − 22.56(6.3) = 97%

3a.

$\sqrt[9]{\dfrac{(46/28)(78/66)(64/58)(26/25)}{(88/63)(39/34)(14/11)(46/35)(26/19)}} =$

$\sqrt[9]{(1.64)(1.18)(1.10)(1.04)(1.39)(1.15)(1.27)(1.31)(1.37)} = 1.26$

3b. Amino acid score = 1.04

3c. PDCAAS = amino acid score × true digestibility = 1.04 (0.87) = 0.905

4. Construct a standard curve % Transmission (y-axis) versus tryptophan (µg) (x-axis). From the standard curve,

estimate the concentration of the tryptophan in the two dilutions:

0.2 ml of diluted sample ⇒ ~1.5 µg of tryptophan

0.5 ml of diluted sample ⇒ ~3.3 µg of tryptophan

Average is 7.0 µg/ml.

Concentration is 7.0 µg of tryptophan/ml × 50 ml/g of supplement = 350 µg of tryptophan/g of supplement.

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18 chapter

Vitamin Analysis

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18.1 INTRODUCTION

18.1.1 Definition and Importance

Vitamins are defined as relatively low-molecular-weight compounds that humans, and for that matter, any living organisms that depend on organic matter as a source of nutrients, require in small quantities for normal metabolism. With few exceptions, humans cannot synthesize most vitamins and need to obtain them from food and supplements. Insufficient levels of vitamins result in deficiency diseases, for example, scurvy and pellagra, which are due to the lack of ascorbic acid and niacin, respectively.

18.1.2 Importance of Analysis

Vitamin analysis of food and other biological samples has played a critical role in determining animal and human nutritional requirements. Furthermore, accurate food composition information is required to determine dietary intakes to assess diet adequacy and improve human nutrition worldwide. From the consumer and industry points of view, reliable assay methods are required to ensure accuracy of food labeling. This chapter provides an overview of techniques for analysis of the vitamin content of food and some of the problems associated with these techniques.

18.2 METHODS

18.2.1 Overview

Vitamin assays can be classified as follows:

1. Bioassays involving humans and animals.
2. Microbiological assays making use of protozoan organisms, bacteria, and yeast.
3. Physicochemical assays that include spectrophotometric, fluorometric, chromatographic, enzymatic, immunological, and radiometric methods.

In terms of ease of performance, but not necessarily with regard to accuracy and precision, the three systems follow the reverse order. It is for this reason that bioassays, on a routine basis at least, are limited in their use to those instances in which no satisfactory alternative methods are available.

The selection criteria for a particular assay depend on a number of factors, including accuracy and precision, but also economic factors and the sample load to be handled. Applicability of certain methods for a particular matrix also needs to be considered. It is important to bear in mind that many official methods presented by AOAC International (1) are limited in their applicability to certain matrices, such as vitamin concentrates, milk, or cereals, and thus cannot be applied to other matrices without some procedural modifications, if at all.

Because of the sensitivity of some vitamins to adverse conditions such as light, oxygen, pH, and heat, proper precautions need to be taken to prevent any deterioration throughout the analytical process, regardless of the type of assay used. Such precautionary steps need to be followed with the test material in bioassays throughout the feeding period. They are required with microbiological and physicochemical methods during extraction as well as during the analytical procedure.

Just as with any type of analysis, proper sampling and subsampling as well as the preparation of a homogeneous sample are critical aspects of vitamin analysis. General guidelines regarding this matter are provided in Chapter 5 of this text.

The principles, critical points, procedures, and calculations for various vitamin analysis methods are described in this chapter. Many of the methods cited are official methods of AOAC International (1). Refer to these methods and other original references cited for detailed instructions on procedures.

18.2.2 Extraction Methods

With the exception of some biological feeding studies, vitamin assays in most instances involve the extraction of a vitamin from its biological matrix prior to analysis. This generally includes one or several of the following treatments: heat, acid, alkali, solvents, and enzymes.

In general, extraction procedures are specific for each vitamin and designed to stabilize the vitamin. In some instances, some procedures are applicable to the combined extraction of more than one vitamin, for example, for thiamin and riboflavin and some of the fat-soluble vitamins (1,2). Typical extraction procedures are:

- Ascorbic acid: Cold extraction with metaphosphoric acid/acetic acid
- Vitamins B_1 and B_2: Boiling or autoclaving in acid plus enzyme treatment
- Niacin: Autoclaving in acid (noncereal products) or alkali (cereal products)
- Vitamin A, E, or D: Organic solvent extraction, saponification, and reextraction with organic solvents. For unstable vitamins such as these, antioxidants are routinely added to inhibit oxidation.

Analysis of fat-soluble vitamins may require saponification, generally either overnight at room temperature or by refluxing at 70°C. In the latter case, an air-cooled

reflux vessel as shown in Fig. 18-1 provides excellent control of conditions conducive to oxidation.

18.2.3 Bioassay Methods

Outside of vitamin bioavailability studies, bioassays at the present are used only for the analysis of Vitamins B₁₂ and D. For the latter it is the reference standard method of analysis of food materials (AOAC Method 936.14), known as the line test (Fig. 18-2), based on bone calcification. Since the determination of Vitamin D involves deficiency studies as well as sacrificing the test organisms, it is limited to animals rather than humans as test organisms.

18.2.4 Microbiological Assays

18.2.4.1 Applications

Microbiological assays are limited to the analysis of water-soluble vitamins. The methods are very sensitive and specific for each vitamin. With certain biological matrices, they are the only feasible methods for some vitamins or matrices. The methods are somewhat time consuming, and strict adherence to the analytical protocol is critical for accurate results.

18.2.4.2 Principle

The growth of microorganisms is proportional to their requirement for a specific vitamin. Thus, in microbiological assays the growth of a certain microorganism in an extract of a vitamin-containing sample is compared with the growth of this microorganism in the presence of known amounts of that vitamin. Bacteria, yeast, or protozoans are used as test organisms. Growth can be measured in terms of turbidity, acid production, gravimetry, or by respiration. With bacteria and yeast, turbidimetry is the most commonly used system. If turbidity measurements are involved, clear sample and standard extracts, versus turbid ones, are essential. In terms of incubation time, turbidity measurement also is a less time-consuming method. The microorganisms are specified by ATCC numbers and are available from the American Type Culture Collection (ATCC) (12301 Parkway Drive, Rockville, MD 20852).

18.2.4.3 Niacin

The procedural sequence for the microbiological analysis of niacin is outlined in Fig. 18-3 [AOAC Method 944.13 and (3)]. Lactobacillus plantarum (ATCC 8014) is the test organism. A stock culture needs to be prepared and maintained by inoculating the freeze-dried culture on bacto-lactobacilli agar and incubating at 37°C for 24

18-1 Reflux vessel useful for saponification.
figure

hr prior to sample and standard inoculation. A second transfer may be advisable in the case of poor growth of the inoculum culture.

In general growth is measured by turbidity. If lactobacilli are used as the test organism, acidimetric measurements can be used as well. The latter may be necessary if a clear sample extract cannot be obtained prior to inoculation and incubation (which is a prerequisite for turbidimetry). In making a choice between the two methods of measurement, one needs to bear in mind the prolonged incubation period of 72 hr that is required with acidimetry.

18.2.4.4 Folate

Folate is the general term including folic acid (pteroylglutamate, PteGln) and poly-γ-glutamyl conjugates with the biological activity of folic acid. Folates present a diverse array of compounds that vary by oxidation state of the pteridine ring structure, one-carbon moieties carried by the specific folate, and the number of conjugated glutamate residues on the folate. Folates are labile to oxidation, light, thermal losses, and leaching when foods are processed. Because of the presence

VITAMIN D BIOASSAY PROCEDURE
Sample Preparation

AOAC International provides specific instructions for preparation of various matrices for the bioassay. In some cases, saponification is used.

Depletion Period

Rats are suitable for depletion at age ≤30 days with body weight of ≥ 44 g but ≤ 60 g. Rachitogenic diet is fed for 18–25 days.

Assay Period

The assay period is the interval of life of the rat between the last day of the depletion period and the eighth or eleventh day thereafter. Feeding protocols are specified. During the assay, depleted rats are fed known and unknown amounts of Vitamin D from standards and samples, respectively.

Potency of Sample

Vitamin D in the sample is determined by the line test from staining of the proximal end of the tibia or distal end of the radius or ulna.

18-2 figure
The bioassay of Vitamin D by the line test, AOAC Method 936.14 45.3.01 (1).

NIACIN MICROBIOLOGICAL ASSAY PROCEDURE
Sample Preparation

Weigh out enough sample to contain ca. 0.1 mg of niacin, add 1 N H_2SO_4, macerate, autoclave 1 hr at 121°C, and cool. Adjust pH to 6.8, dilute to volume (ca. 0.1 g niacin/ml), mix, and filter.

Assay Tube Preparation

In at least duplicate use 0.0, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 ml sample filtrate and make up the difference to 5.0 ml with H_2O, then add 5.0 ml of Difco Basal Medium for Niacin Assay broth to each tube, autoclave 10 min at 121°C, and cool.

Standard Preparation

Prepare assay tube in at least duplicate using 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, and 5.0 ml standard solution (0.1 μl/ml niacin), make up difference to 5.0 ml with H_2O, then add 5.0 ml of assay broth and treat identically to sample tubes.

Inoculation and Incubation

Prepare inoculum using *Lactobacillus plantarum*, ATCC 8014 in Bacto Lactobacilli Broth AOAC (Difco). Add one drop of inoculum to each tube, cover tubes, and then incubate at 37°C for 16–18 hr, i.e., until maximum turbidity is reached in tubes containing the highest concentration of niacin.

Determination

Measure %T or absorbance at any wavelength between 540 and 660 nm.

18-3 figure
The microbiological assay of niacin, AOAC Method 944.13, 45.2.04 (1).

of multiple forms in food products and its instability, folate presents a difficult analytical problem. Total folate can be assayed microbiologically; however, to specifically quantitate folate forms present in a biological matrice, liquid chromatographic procedures must be used.

The microbiological assay follows for total folate in foods by microbiological assay by *Lactobacillus rhamnosus* and trienzyme digestion (4).

18.2.4.4.1 Principle Folate in the sample is extracted in buffer at 100°C (boiling water bath). The extract is digested with a conjugase (to cleave poly-γ-glutamyl folates to PteGln₃ or lower), and α-amylase and protease (to free macromolecularly bound folates). Growth response of the assay microorganism is measured by percent transmittance. Transmittance depends on folate concentration.

18.2.4.4.2 Critical Points Care must be taken to protect labile folates from oxidation and photochemical degradation. Reducing agents including ascorbic acid, β-mercaptoethanol, and dithiothreitol are effective in preventing oxidation. Strict adherence to microbiological assay techniques is necessary to assay folate with accuracy and precision.

18.2.4.4.3 Procedure Figure 18-4 gives the procedural steps of the assay.

18.2.4.4.4 Calculations Results are calculated manually or from the regression line of the standard curve responses using 4th degree polynomial plots and a computer program written to conform to AOAC microbiological analysis protocol. Results are reported as milligrams of vitamin per 100 g or per serving.

18.2.5 Physicochemical Methods

18.2.5.1 Vitamin A

Vitamin A is sensitive to ultraviolet (UV) light, air (and any prooxidants, for that matter), high temperatures, and moisture. Therefore, steps need to be taken to avoid any adverse changes in this vitamin due to such effects by using low actinic glassware, nitrogen and/or vacuum, as well as avoiding excessively high temperatures. The addition of an antioxidant at the onset of the procedure is highly recommended. High performance liquid chromatographic (HPLC) methods are considered the only acceptable methods to provide accurate food measurements of Vitamin A activity.

Details follow of the HPLC method for Vitamin A (retinol isomers) in milk and milk-based infant formula (AOAC Method 992.04, 50.1.02) (1):

18.2.5.1.1 Principle The sample is saponified, the Vitamin A (retinol) extracted into organic solvent and concentrated, and all-*trans*-retinol and 13-*cis*-retinol levels are determined by HPLC on a silica column.

18.2.5.1.2 Critical Points All work must be in subdued artificial light. Care must be taken to avoid oxi-

FOLATE MICROBIOLOGICAL ASSAY PROCEDURE
Sample Preparation

To 1.2–2.0 g of sample, add 50 ml of specified buffer, homogenize, and proceed to digestion step. (High fat samples should be extracted with hexane, and all samples should be protected from light and air.)

Trienzyme Digestion

Boil samples for 5 min. and cool to room temperature. Digest each sample in sequence with specified conjugase, α-amylase, and protease. Deactivate enzymes by boiling for 5 min. Cool tubes, filter, and dilute an appropriate aliquot to a final concentration of ca. 0.15 ng/mL.

Preparation of Standard Curve and Blank Tubes

Construct an 8-point standard curve using a working standard solution of folate. Add 5 ml of *Lactobacillus casei* assay medium to each tube. Prepare an uninoculated blank and an inoculated blank to zero the spectrophotometer, and an enzyme blank to determine the contribution of the enzymes to microbial growth.

Assay

Folic acid is assayed by growth of *L. rhamnosus* according to AOAC Method 960.46 (1). Prepared tubes of the samples, standard curve, inoculated and uninoculated blanks, and enzyme blank are autoclaved at 121°C for 5 min and then inoculated with one drop of prepared inoculum per tube. After tubes are incubated at 37°C for 20–24 hr, growth response is measured by percent transmittance at 550 nm.

18-4 *figure* Analysis of folate in food using *Lactobacillus rhamnosus* ATCC No. 7469 and a trienzyme extraction procedure. [Adapted from (4).] Refer to (4) for more details on procedure.

VITAMIN A HPLC ANALYSIS PROCEDURE
Sample Preparation

Transfer 40 ml of ready-to-feed formula or fluid milk into a 100-ml digestion flask. Add 10 ml of ethanolic pyrogallol solution (2% pyrogallol in 95% ethanol) and saponify with ethanolic KOH (10% KOH in 90% ethanol) at room temperature for 18 hr or at 70°C using the reflux vessel in Fig. 18-1.

Extraction of Digest

Pipet 3 ml of digest into 15-ml centrifuge tube and add 2 ml of water. Extract with 7 ml hexane–diethylether (85 + 15). Repeat extraction 2× with 7-ml portions of extractant. Pool extracts in a 25-ml volumetric flask. Add 1 ml of hexadecane solution [hexadecane + hexane (1 + 100)] and dilute to volume with hexane. Pipet 15 ml of diluted extract into a centrifuge tube and evaporate under nitrogen. Dissolve residue in 0.5 ml of heptane.

Chromatography Parameters

Column	15 cm × 4.5 mm packed with 3 mm silica (Apex 3 μm silica)
Mobile Phase	Isocratic, heptane, and isopropanol (1–5%)
Detection	UV, 340 nm
Flow Rate	1–2 ml/min

(Exact mobile phase composition and flow rate are determined by system suitability test to give retention times of 4.5 and 5.5 min for 13-*cis*-retinol and all-*trans*-retinol, respectively.)

18-5 *figure* The HPLC analysis of Vitamin A in milk and milk-based infant formula, AOAC Method 992.05, 50.1.02 (1).

dation of the retinol during the entire procedure. Solvent evaporation should be completed under nitrogen, and hexadecane is added to prevent destruction during solvent evaporation.

18.2.5.1.3 Procedure Figure 18-5 gives the procedural steps of the assay. Pyrogallol is added prior to saponification as an antioxidant.

18.2.5.1.4 Calculations

$$\text{all-}trans\text{-retinol (mg/ml)} = \frac{A_T/A_{ST} \times C_T \times DF}{V_{SPI}} \quad [1]$$

where:

A_T = peak area, all-*trans*-retinol in sample
A_{ST} = peak area, all-*trans*-retinol in standard
C_T = concentration, all-*trans*-retinol (mg/ml)/
V = sample volume (ml)
DF = dilution factor

$$13\text{-}cis\text{-retinol (μg/ml)} = \frac{A_C/A_{SC} \times C_C \times DF}{V_{SPI}} \quad [2]$$

where:

A_C = peak area, 13-*cis*-retinol in sample
A_{SC} = peak area, 13-*cis*-retinol in standard
C_C = concentration 13-*cis*-retinol (mg/ml)
V = sample volume (ml)
DF = dilution factor

18.2.5.2 Vitamin E (Tocopherols and Tocotrienols)

18.2.5.2.1 Vitamin E Compounds Vitamin E is present in foods as eight different compounds. All are 6-hydroxychromans. The Vitamin E family is comprised of α-, β-, γ-, and δ-tocopherol, characterized by a saturated side chain of three isoprenoid units and the corresponding unsaturated tocotrienols (α-, β-, γ-, and δ-). All homologs in nature are (R, R, R)-isomers. The National Research Council defined dietary Vitamin E activity in terms of (R, R, R)-α-tocopherol equivalents (α-TE). One α-TE is the activity of 1 mg of (R, R, R)-α-T. For mixed diets containing only natural forms of Vitamin E, currently accepted factors for the conversion of the tocopherols and tocotrienols to α-TE units are the following:

α-tocopherol—g × 1.0
β-tocopherol—mg × 0.5
γ-tocopherol—mg × 0.1
δ-tocopherol—mg × 0.03
α-tocotrienol—mg × 0.3
β-tocotrienol—mg × 0.05
γ-tocotrienol—unknown
δ-tocotrienol—unknown

synthetic α-tocopherol—mg × 0.74
synthetic α-tocopheryl acetate—mg × 0.67

Since natural Vitamin E occurs in multiple forms with varying biological activities, estimation of its activity requires quantitation of each form. The accepted methodology is by HPLC.

Details follow of Vitamin E analysis in food products using HPLC (5).

18.2.5.2.2 Principle

1. General food products. The sample is saponified under reflux (see Fig. 18-1), extracted with hexane, and injected onto a normal phase HPLC column connected to a fluorescence detector, $Ex \lambda = 290$ nm, $Em \lambda = 330$ nm.

2. Margarine and vegetable oil spreads. The sample is dissolved in hexane, $MgSO_4$ is added to remove water, and the filtered extracts are assayed by HPLC (see 1).

3. Oils. Oil is dissolved in hexane and injected directly onto an HPLC column.

18.2.5.2.3 Critical Points
Vitamin E is subject to oxidation. Saponification is completed under reflux, in the presence of the antioxidant, pyrogallol, with the reaction vessel protected from light.

18.2.5.2.4 Procedure
The Vitamin E assay is detailed in Fig. 18-6 and an example chromatogram is given in Fig. 18-7.

18.2.5.2.5 Calculations
Vitamin E is quantitated by external standards from peak area by linear regression.

18.2.5.3 Vitamin C

The vitamin (l-ascorbic acid and l-dehydroascorbic acid) is very susceptible to oxidative deterioration, which is enhanced by high pH and by the presence of ferric and cupric ions. For these reasons, the entire analytical procedure needs to be performed at low pH and, if necessary, in the presence of a chelating agent.

Mild oxidation of ascorbic acid results in the formation of dehydroascorbic acid that also is biologically active and that is reconvertible to ascorbic acid by treatment with reducing agents, including β-mercaptoethanol and dithiothreitol.

18.2.5.3.1 2, 6-Dichloroindophenol Titrimetric Method (AOAC Method 967.21, 45.1.14) (1, 6)

1. Principle. L-ascorbic acid is oxidized to L-dehydroascorbic acid by the indicator dye. At the endpoint, excess unreduced dye is rose-pink in acid solution. L-Dehydroascorbic acid can be determined by first con-

VITAMIN E HPLC ANALYSIS PROCEDURE
Sample Preparation

a. General food products: Add 10 ml of 6% (wt/vol) pyrogallol in ethanol to sample, mix, and flush with N_2. Heat at 70°C for 10 min with sonication. Add 2 ml of 60% KOH solution, mix, and flush with N_2. Digest for 30 min at 70°C. Sonicate for 5 min. Cool to room temperature, and add sodium chloride and water. Extract with hexane (0.1% BHT) three times. Combine hexane extracts. Add 0.5 g of $MgSO_4$ and mix. Filter through a Millipore filtration apparatus (0.45 μm). Dilute to volume with hexane. Inject 20 μl.

b. Margarine and vegetable oil spreads: Add 40 ml of hexane (0.1% BHT) to a 10-g sample and mix. Add 3 g of $MgSO_4$, mix, let stand ±two hr. Filter and dilute combined filtrate to volume with hexane (0.1% BHT). Inject 20 μl.

Chromatography Parameters
Column—Hibar RT, Lichrosorb Si60 5 μm, 25.0 cm × 4.6 mm
Mobile phase—0.9% isopropanol in hexane
Flow—1 ml/min
Detector—Fluorescence, $Ex \lambda = 290$ nm, $Em \lambda = 330$ nm
(Note: Determine recovery for each food product.)

18-6 figure Analysis of Vitamin E in food products using HPLC. [Adapted from (5).] Refer to (5) for details on applications.

verting it to L-ascorbic acid with a suitable reducing agent.

2. Procedure. Figure 18-8 outlines the protocol followed with this method. In the presence of significant amounts of iron or copper in the biological matrix to be analyzed, it is advisable to include a chelating agent such as EDTA (ethylenediaminetetraacetic acid) with the extraction.

The red-color endpoint should last at least 10 sec to be valid. With colored samples such as red beets or heavily browned products, the endpoint is impossible to detect by human eyes. Therefore, in such cases it needs to be determined by observing the change of transmittance using a spectrophotometer with the wavelength set at 545 nm.

3. Calculation.

$$\text{mg of ascorbic acid/g or ml of sample} = C \times V \times (DF/WT) \quad [3]$$

where:

C = mg of ascorbic acid/ml of dye
V = ml of dye used for titration of diluted sample
DF = dilution factor
WT = sample weight, g

18.2.5.3.2 Microfluorometric Method (AOAC Method 967.22, 45.1.15) (1, 7)

1. Principle. This method measures both ascorbic acid and dehydroascorbic acid. Ascorbic acid, following oxidation to dehydroascorbic acid, upon reaction
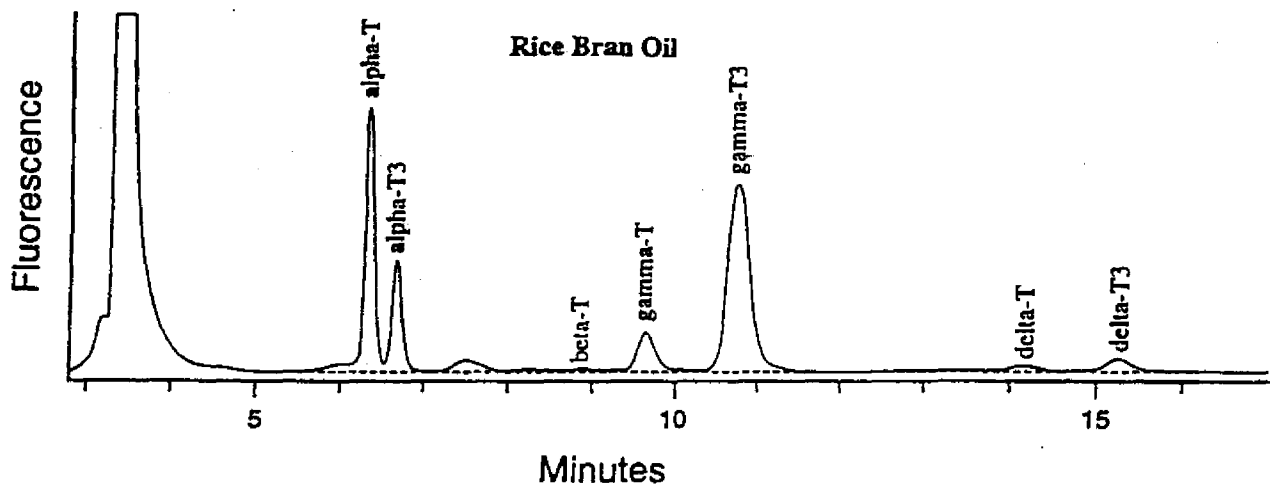
18-7
figure

Chromatogram of rice bran oil showing tocopherols and tocotrienols.

VITAMIN C ASSAY PROCEDURE
2, 6-DICHLOROINDOPHENOL TITRATION
Sample Preparation

Weigh and extract by homogenizing sample in metaphosphoric acid–acetic acid solution (15 g of HPO_4 and 40 ml of HOAc in 500 ml of H_2O). Filter (and/or centrifuge) sample extract and dilute appropriately to a final concentration of 10–100 mg of ascorbic acid/100 ml.

Standard Preparation

Weigh 50 mg of l-ascorbic acid and dilute to 100 ml with metaphosphoric acid–acetic acid extracting solution.

Titration

Titrate three replicates each of standard and sample with dichloroindophenol solution to a pink endpoint lasting at least 10 sec.

18-8
figure

Analysis of Vitamin C by the 2, 6-dichloroindophenol titration, AOAC Method 967.21, 45.1.14 (1). [Adapted from (6).]

with o-phenylenediamine, forms a fluorescent quinoxaline compound.

2. Procedure. The procedural sequences for this method are outlined in Fig. 18-9. To compensate for the presence of interfering extraneous material, blanks need to be run using boric acid prior to the addition of the diamine solution.

3. Calculations.

$$\text{mg of ascorbic acid/g or ml} =$$
$$[(A - B)]/(C - D)] \times S \times DF/WT \qquad [4]$$

where:

A and C = fluorescence of sample and standard, respectively

VITAMIN C MICROFLUOROMETRIC ASSAY PROCEDURE
Sample Preparation

Prepare sample and extract as outlined in Fig. 18-8. To 100 ml each of standard and sample solution add 2 g of acid washed Norit, shake vigorously, and filter.

Blank Preparation

Transfer 5 ml of each filtrate to separate 100-ml volumetric flasks containing 5 ml of H_3BO_3–NaOAC solution. Let stand 15 min, swirling occasionally. Designate as standard or sample blank. Development of fluorescent quinoxaline is prevented by the formation of H_3BO_3–dehydroascorbic acid complex prior to addition of phenylenediamine solution. Transfer 2 ml of each solution to each of three tubes.

Sample Determination

Transfer 5 ml of each standard and sample solution to 100-ml volumetric flasks, each containing 5 ml of 50% NaOAc trihydride and ca. 27 ml of H_2O, and dilute to volume with H_2O, then transfer 2 ml of standard and sample solution to each of two sets of three fluorescence tubes.

Formation of Quinoxaline

To sample and blank tubes, add 5 ml of 20 mg% aq. o-phenylenediamine solution to each tube, swirl using a Vortex mixer, and allow to stand for 35 min at room temperature.

Determination

Measure fluorescence at Ex = 356 nm, Em = 440 nm.

18-9
figure

Analysis of Vitamin C by the microfluorometric method, AOAC Method 967.22, 45.1.15 (1). [Adapted from (7).]

B and D = fluorescence of sample and standard blanks, respectively
S = concentration of standard in mg/ml
DF = dilution factor
WT = sample weight, g

18.2.5.4 Thiamin (Vitamin B₁) in Foods, Thiochrome Fluorometric Procedure (AOAC Method 942.23, 45.1.05) (1)

18.2.5.4.1 Principle This method is based on the fluorescence measurement of the oxidized form of thiamin, thiochrome, following extraction and enzymatic hydrolysis of the phosphate esters of thiamin and chromatographic cleanup.

18.2.5.4.2 Critical Points Thiochrome is light sensitive. Therefore, the analytical steps following the oxidation must be performed under subdued light. Thiamin is sensitive to heat, especially at alkaline pH. The analytical steps starting with the oxidation of thiamin through the fluorescence measurement (Fig. 18-10) need to be carried out rapidly and precisely according to the instructions.

18.2.5.4.3 Procedure Figure 18-10 outlines the procedural sequence of the thiamin analysis. The enzymatic treatment or the chromatographic cleanup may not be necessary with certain matrices, such as vitamin concentrates that contain nonphosphorylated thiamin and no significant amounts of substances that could interfere with the determination.

THIAMIN ANALYSIS BY THIOCHROME PROCEDURE
Sample Preparation

Weigh out sample that contains ca. 10–20 µg of thiamin, add HCl, mix, autoclave for 15 min at 121°C, then cool. Adjust pH to 4.5–5.0 with HCl, add enzyme solution, and incubate 3 hr at 45–50°C. Cool sample, adjust to pH 3.5, dilute to volume with water, mix, and filter. Subject standard solution of thiamin to same enzyme treatment.

Enzyme Hydrolysis

Add enzyme solution to samples and standards, mix, and incubate 3 hr at 45–50°C. Cool, adjust to pH 3.5, dilute to volume with water, and filter.

Sample Extract Cleanup

Apply sample extract to specified ion-exchange column, wash column with hot water, then elute thiamin with hot acid-KCl solution. Cool eluted sample and dilute to volume with acid-KCl solution. Treat standards identically.

Thiochrome Formation

Convert thiamin to thiachrome using $K_2Fe(CN)_6$, add isobutyl alcohol, shake vigorously, and centrifuge. Decant isobutyl alcohol fraction into fluorescence reading tube, and read at 365 nm/435 nm Ex/ Em. Treat standard solution identically, read at 365 nm/435 nm Ex/Em, and develop standard curve to quantitate samples.

18-10
figure

Analysis of thiamin by the thiochrome fluorometric procedure, AOAC Method 942.2.3, 45.1.05 (1). Refer to (1) for more details on procedure.

18.2.5.4.4 Calculations

$$\mu g \text{ of thiamin/g} =$$
$$(S - S_b/Std - Std_b) \times C/A \times 25/V_p \times V_o/WT \quad [5]$$

where:

S and S_b = fluorescence of sample and sample blank, respectively

Std and Std_b = fluorescence of standard and standard blank, respectively

C = concentration of standard, g/ml
A = aliquot taken, ml
25 = final volume of column eluate, ml
V_p = volume passed through chromatography column, ml
V_o = dilution volume of original sample, ml
WT = sample weight, g

18.2.5.5 Riboflavin (Vitamin B₂) in Foods and Vitamin Preparations, Fluorometric Method (AOAC Method 970.65, 45.1.08) (1)

18.2.5.5.1 Principle Following extraction, cleanup, and compensation for the presence of interfering substances, riboflavin is determined fluorometrically.

18.2.5.5.2 Critical Points Because of extreme sensitivity of the vitamin to UV light, all operations need to be conducted under subdued light. The analyst also needs to be aware that exact adherence to the permanganate oxidation process is essential for reliable results.

18.2.5.5.3 Procedure An outline of the procedural protocol for this analysis is shown in Fig. 18-11. In spite of the fact that riboflavin is classified as a water-soluble vitamin, it does not easily dissolve in water. When preparing the standard solution, the analyst needs to pay special attention that the riboflavin is completely dissolved.

18.2.5.5.4 Calculations

$$\text{mg of riboflavin/g of sample} = [(A - C)/(B - A)] \quad [6]$$
$$\times (CS/V)$$
$$\times (DF/WT)$$

where:

A and C = fluorescence of sample containing water and sodium hydrosulfite, respectively
B = fluorescence of sample containing riboflavin standard
CS = concentration of standard, mg/ml
V = volume of sample for fluorescence measurement, ml

RIBOFLAVIN ASSAY PROCEDURE BY FLUORESCENCE
Sample Preparation

Weigh out homogenized sample, add 0.1 N HCl, mix, then autoclave for 30 min at 121°C and cool. Precipitate interfering substances by adjusting pH to 6.0 immediately followed by a pH readjustment to 4.5, dilute to volume with H_2O, and filter.

Oxidation of Interfering Materials

Oxidize as follows: Place 10 ml of filtrate into each of four tubes. To two of the tubes add 1.0 ml of H_2O, and add 1.0 ml of standard solution (0.5 μg/ml of riboflavin) to each of the two remaining tubes. To each tube, one at a time, add 1.0 ml of glacial HOAc, followed by 0.5 ml of 3% $KMnO_4$; allow to stand for 2 min; then add 0.5 ml of 3% H_2O_2. Shake well.

Measurement of Fluorescence

Measure fluorescence at $Ex \lambda = 440$, $Em \lambda = 565$. First read sample extracts containing H_2O, then add 20 mg $Na_2S_2O_4$ and mix and reread. Next read standard samples.

18-11 Analysis of riboflavin by fluorescence, AOAC
figure   Method 970.65, 45.1.08 (1).

DF = dilution factor
WT = weight of sample, g

18.3 COMPARISON OF METHODS

Each type of method has its advantages and disadvantages. In selecting a certain method of analysis for a particular vitamin or vitamins, a number of factors need to be considered, some of which are listed below:

1. Method accuracy and precision
2. The need for bioavailability information
3. Time and instrumentation requirements
4. Personnel requirements
5. The type of biological matrix to be analyzed
6. The number of samples to be analyzed
7. Regulatory requirements—Must official AOAC International methods be used?

Bioassays are extremely time consuming. Their uses generally are limited to those instances in which no suitable alternate methods are available, or in cases in which bioavailability of the analyte is desired, especially if other methods have not been demonstrated to provide this information. Bioassays have the advantage that they sometimes do not require the preparation of an extract, thus eliminating the potential of undesirable changes of the analyte during the extract preparation. On the other hand, in the case of deficiency development requirements prior to analysis, bioassays are limited to animals rather than humans.

Both microbiological and physicochemical methods require vitamin extraction, i.e., solubilization prior to analysis. In general, the results obtained through

these methods represent the total content of a particular vitamin in a certain biological matrix, such as food, and not necessarily its bioavailability to humans.

The applicability of microbiological assays is limited to water-soluble vitamins. While somewhat time consuming, they generally can be used for the analysis of a relatively wide array of biological matrices without major modifications. Furthermore, less sample preparation is often required compared to physicochemical assays.

Because of their relative simplicity, accuracy, and precision, the physicochemical methods, in particular the chromatographic methods using HPLC, are preferred. While HPLC involves a high capital outlay, it is applicable to most vitamins and lends itself in some instances to simultaneous analysis of several vitamins and/or vitamers (isomers of vitamins). However, although its applicability has been demonstrated in some cases to a wide variety of biological matrices with no or only minor modifications, one must always bear in mind that all chromatographic methods, including HPLC, are separation and not identification methods. Therefore, during adaptation of an existing HPLC method to a new matrix, establishing evidence of peak identity and purity is an essential step of the method adaptation or development.

When selecting a system for analysis, at least initially, it is wise to consider the use of official methods that have been tested through interlaboratory studies and that are published by such organizations as AOAC International (1) or the American Association of Cereal Chemists (AACC) (8). Again, one must realize that these methods are limited to certain biological matrices.

Recent developments regarding new methods involve mostly HPLC systems (2,9–11). To keep current with new developments in the area of vitamin analysis, the journal Food Chemistry is an excellent reference.

18.4 SUMMARY

The three most used types of methods for the analysis of vitamins—bioassays and microbiological and physicochemical assays—have been outlined in this chapter. They are, in general, applicable to the analysis of more than one vitamin and several food matrices. However, the analytical procedures need to be properly tailored to the analyte and the biological matrix to be analyzed, including sample preparation, extraction, and quantitative measurements. It is essential to validate any new application appropriately by assessing its accuracy and precision. Method validation is especially important with the chromatographic methods such as HPLC, since basically these methods accent separations rather than identification of compounds.

For this reason, it is essential to ensure not only identity of these compounds but, just as important, their purity.

18.5 STUDY QUESTIONS

1. What factors should be considered in selecting the assay for a particular vitamin?
2. To be quantitated by most methods, vitamins must be extracted from foods. What treatments are commonly used to extract the vitamins? For one fat-soluble vitamin and one water-soluble vitamin, give an appropriate extraction procedure.
3. What two vitamins must be listed on the standard nutritional label? What vitamins were listed on the old version of the nutritional label but are no longer required on the current standard nutritional label? (See Chapter 3.)
4. The standard by which all chemical methods to measure Vitamin D content are compared is a bioassay method. Describe this bioassay method.
5. Explain why it is possible to use microorganisms to quantitate a particular vitamin in a food product, and describe such a procedure.
6. Niacin and folate both can be quantitated by microbiological methods. What extra procedures and precautions are necessary in the folate assay compared to the niacin assay, and why?
7. There are two commonly used AOAC methods to measure the Vitamin C content of foods. Identify these two methods; then compare and contrast them with regard to the principles involved.
8. During processing and storage of foods, L-ascorbic acid can be oxidized to L-dehydroascorbic acid. Using the 2, 6-dichloroindophenol titrimetric for Vitamin C, how could you quantitate total Vitamin C and each form individually?
9. What are the advantages and disadvantages of using HPLC for vitamin analysis?

18.6 PRACTICE PROBLEMS

Calculate the concentration of vitamin in the original sample for each of the vitamin and assay conditions described below.

1. Niacin, microbiological assay
   - Sample weight: 0.1120 g
   - Dilutions: 1:100 and 0.1:500
   - Niacin concentration in sample: 0.86 μg/ml
2. Vitamin C, dichloroindophenol method
   - Sample weight: 100 g, diluted to 500 ml with extracting solution
   - Amount of sample filtrate titrated: 25 ml
   - Amount of dye used in titration: 9.1 ml

- Ascorbic acid concentration in dye: 0.175 mg/ml
3. Thiamin, fluorometric method
   - Sample weight: 2.0050 g
   - Dilutions: Dilute to 100 ml, take 25 ml for chromatography, then dilute eluate to 25 ml and use 5 ml for fluorometry
   - Standard concentration: 0.1 μg/ml
   - Fluorometry reading ratio: 0.850
4. Riboflavin, fluorometric method
   - Sample weight: 1.0050 g
   - Dilutions: to 50 ml; use 10 ml for fluorometry
   - Fluorometry readings: $A_{60}/B_{35}/C_{10}$
   - Riboflavin concentration: 0.1 μg/ml

Answers

1. 4.3 mg/g; 2. 0.32 mg/g; 3. 0.8479 mg/g; 4. 0.9950 mg/g

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19

chapter
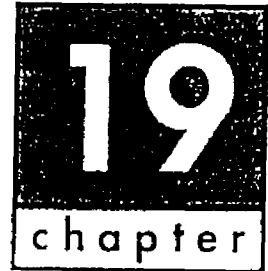
Pigment Analysis

Steven J. Schwartz

19.1 INTRODUCTION

19.1.1 Importance of Color and Food Quality

Color is one of the most important quality attributes of foods. The first impression of the quality and acceptability of a particular food is judged upon its appearance. Therefore, the pigments, which are the prime contributors to coloration, are important quality constituents to analyze in foods.

Measurement of both natural and synthetic pigments in foods is an analytical challenge to food chemists. The diversity of naturally occurring pigments, their derivatives, and the formation of degradation components that contribute to the color of foods complicate both qualitative and quantitative measurements. Furthermore, compartmentalization of natural pigments within foods and interactions that may occur between pigments and other food components, especially during thermal processing, may lead to difficulties in liberating or extracting the pigments for analysis. Despite these obstacles, a number of excellent methods have been developed specifically for the extraction, separation, and measurement of pigments in foods. This chapter summarizes some of the current methods used for this analysis with applications aimed toward methodology appropriate for use in a food analysis course.

19.1.2 Presence and Distribution of Pigments in Foods

Basically, there are five major classes of naturally occurring pigments in foods. Specifically, four pigment classes are distributed throughout the plant kingdom and the fifth in animal tissues. The lipid-soluble chlorophylls and carotenoids and the water-soluble anthocyanins and betalains are found in plants. In animal tissues, meat color is due to a heme protein, myoglobin. In some fish tissue, such as salmon and trout and in crustaceans, the orange-red coloration arises because of the presence of carotenoid pigments. However, carotenoid pigments found in the animal kingdom are not biosynthesized but derived from plant sources.

19.1.3 Basic Principles in Handling and Storage of Pigments

It is well known that naturally occurring pigments and synthetic dyes can be sensitive to oxygen, heat, light, metal ions, and catalysts that enhance the rate of oxidative and reductive reactions. Therefore, care should always be taken to minimize these reactions during extraction, handling, and storage of pigments. Plant tis-

sue pigments can be extracted fresh from the raw state; however, some losses may occur because of enzymatic activity. Plant pigments may be extracted from the frozen tissue; however, prior to freezing, the tissue samples should be blanched to inactivate the native enzymes that can decompose the pigments. Once extracted, the analyst should ensure that contaminant-free glassware always is used and pigment extracts are stored under a nitrogen headspace.

Extracts under nitrogen should be stored under reduced temperature conditions in amber glass vials or clear glass vials wrapped in aluminum foil to prevent exposure to light. Aqueous extracts preferably should not be frozen since freeze concentration can enhance pigment–pigment interactions. However, the storage stabilities of some pigments are enhanced at frozen temperatures, and standard control solutions should be checked regularly for the extent of degradation. Optimally, freshly prepared extracts should be analyzed immediately to minimize chemical alterations and pigment decomposition.

If a quantitative measurement of the pigments is required, all tissues should be subjected to a moisture content analysis. This will provide quantitative data on pigments on both a wet-weight and dry-weight basis. Since the moisture content of tissues may differ from sample to sample and changes during processing, it may be necessary to monitor the changes in solids content to determine if losses in pigment content have occurred.

In the following sections, the chemical properties and techniques involved in the analysis of specific food pigments are discussed. All the methods covered involve spectrophotometric measurements, and therefore, the reader is advised to refer to Chapter 25 on Basic Principles of Spectroscopy and Chapter 26 for a thorough review of ultraviolet and visible spectroscopy. The reader also is referred to Chapter 32 on high performance liquid chromatography and to Chapter 37 on color analysis.

19.2 CHLOROPHYLLS

In higher plants, the chlorophylls, both a and b, are found ubiquitously throughout photosynthetic tissues. In vegetable tissues, a number of different chlorophyll derivatives can be present, especially after thermal processing, that contribute to the overall color of vegetable products. The structures of the chlorophylls and their derivatives found in food products are depicted in Fig. 19-1.

Several analytical methods have been developed for the analysis of chlorophylls in foods. Many of these methods have been compiled in a review by Schwartz and Lorenzo (1). Early spectrophotometric methods
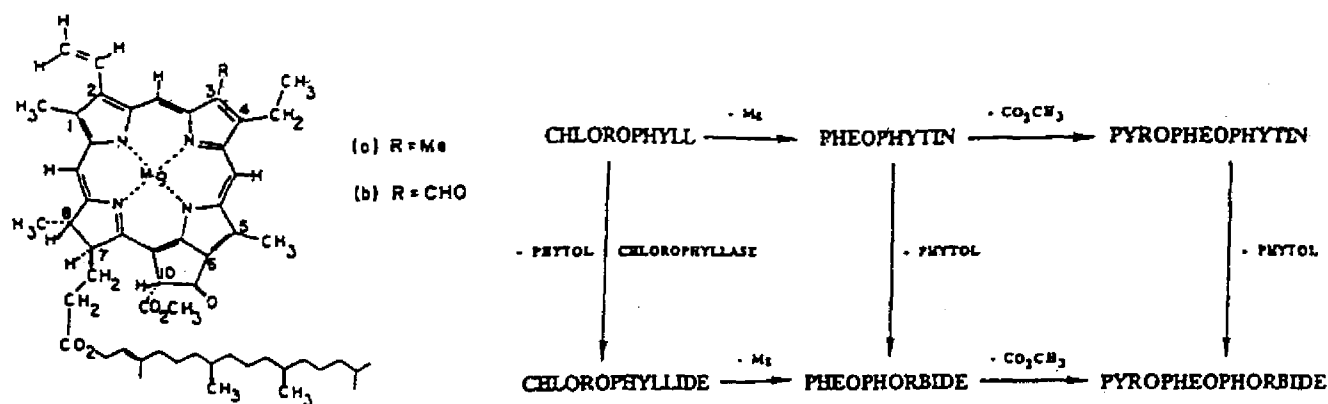
19-1 figure The structure of chlorophyll and its derivatives. (Reprinted with permission from *Critical Reviews in Food Science and Nutrition.* 1990. Vol. 29, No. 1, pp.1–17. Copyright CRC Press, Inc., Boca Raton, FL.)

allowed for the determination of chlorophylls by measuring absorbance at the absorption maxima of the two chlorophylls. These methods are suitable only for fresh plant materials in which no pheophytin degradation components are present. This is the basis for the AOAC International spectrophotometric procedure (2) (Method 942.04), which provides results for total chlorophyll, chlorophyll *a*, and chlorophyll *b* content. Vernon (3) developed a quantitative spectrophotometric method for the analysis of both chlorophylls *a* and *b* as well as pheophytin *a* and *b*. The method utilizes specific absorptivities of the four components to derive a set of equations to calculate the quantities of each individual pigment in 80% acetone solutions. The method generally is applicable to vegetable tissue in which only chlorophyll and pheophytin derivatives are present. High performance liquid chromatography (HPLC) methods are preferred when other chlorophyll components such as chlorophyllides, pheophorbides, and pyropheophytins are expected.

Acetone extracts generally are used for quantitative extraction of chlorophylls from plant tissue. Tissue samples usually are blended in acetone in the presence of a small quantity of $CaCO_3$. The $CaCO_3$ base neutralizes any acids that may be liberated from the tissue and prevents the formation of pheophytins during extraction. Since water always is present in the tissue cells, a final concentration of 20% water and 80% acetone is often used, and therefore, drying of the sample is not necessary. Furthermore, specific absorption coefficients and molar absorptivity values are widely published for 80% acetone solutions, allowing for simple concentration calculations of pure pigment extracts. Acetone extracts of pigments also are generally compatible with reversed phase HPLC methods, which use some water in the mobile phase. Alternatively, chlorophyll pigments can be easily transferred to other organic solvents, such as diethyl ether, by repeated

washings of the acetone–ether mixture with water in a separatory funnel.

A simple reversed-phase HPLC method for the analysis of chlorophylls and their derivatives in fresh and processed plant tissues has been described by Schwartz et al. (4). The method is applicable to determine chemical alterations in chlorophyll composition during the processing of foods and can separate and quantitate the major chlorophyll derivatives including the pheophytins and pyropheophytins. The method involves a gradient elution technique that can be eliminated if chlorophyllides and pheophorbides are not present or found in small quantities (5). Typical HPLC chromatograms of fresh, blanched, frozen, and canned spinach are shown in Fig. 19-2. An advantage of the method is that chromatograms are monitored at 654 nm, which selectively screens for chlorophyll components while the yellow-colored carotenoids are excluded. More sophisticated methods have been published for the separation of the water-soluble chlorophyll components (6,7), as well as for the measurement of both chlorophyll and carotenoids in plant tissue (8) and in oil (9). Identification of the purified chlorophyll pigments present in extracts can be performed by comparison of ultraviolet-visible (UV-Vis) spectra and coelution with authentic standards. Mass spectrometry (see Chapter 29) also has been used to confirm the identity of individual chlorophylls (10,11).

19.3 CAROTENOIDS

The carotenoid pigments consist of two major classes: the hydrocarbon carotenes and the oxygenated xanthophylls. Not only do the carotenoids provide yellow to red coloration in foods, but some also serve as precursors to Vitamin A. For this reason, many analytical methods for carotenoids are aimed at measurement of
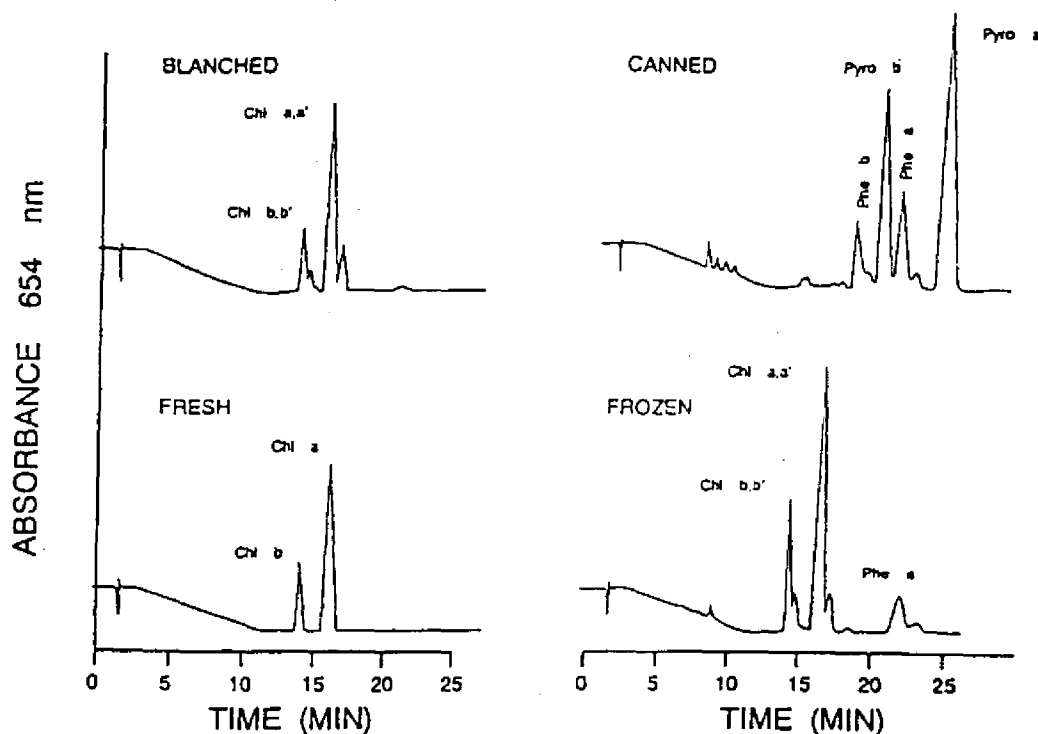
HPLC chromatograms of chlorophylls in fresh, blanched, frozen, and canned spinach. [Reprinted with permission from (4). Copyright 1981 American Chemical Society.]

the provitamin A carotenoids for determination of their nutritional value. In addition to over 500 naturally occurring carotenoids, β-carotene, β-apo-8'-carotenal, canthaxanthin, astaxanthin, and other carotenoids have been synthesized and used as feed ingredients (i.e., poultry and salmon) as well as to enhance the appearance of a variety of manufactured food products. Some of these lipid-soluble carotenoids also are available in water-dispersible forms for use in beverage products. The carotenoids present in annatto, marigold, saffron, tumeric, and paprika, to mention a few, also are used to color foods. The structures of some prominent carotenoids are shown in Fig. 19-3 as examples.

The complex nature and diversity of carotenoid compounds present in plant foods necessitates chromatographic separation. The AOAC Method 941.15 (2) recommends extraction with acetone-hexane, filtration, and removal of the acetone by repeated washings with water. Hexane extracts then are applied to a MgO_2 (activated) diatomaceous earth column and eluted with a mixture of acetone and hexanes. Because most carotenoid extracts consist of a mixture of nonpolar carotenes and more polar xanthophylls, a carotene fraction elutes early from the column in a chromatographic elution. As the acetone concentration is increased, the more polar xanthophylls elute separately as the monohydroxy and dihydroxy pigments. Alternatively, total

xanthophylls can be eluted and isolated by addition of methanol to the eluting solvent. After dilution to volume, the concentrations of carotenes and xanthophylls are measured by absorbance readings based on standard solutions. The major concern about using the AOAC procedure (Method 941.15) is its failure to distinguish between coeluting carotenes: no separation occurs between α- and β-carotene as well as other hydrocarbon carotenoids. The method also calculates all carotenes as β-carotene, which possesses the highest provitamin A activity of all the carotenes. Thus, if the provitamin A activity is to be determined, the results for Vitamin A content can be severely overestimated.

Extraction procedures for quantitative removal of carotenoids from plant tissues involve the use of organic solvents that must penetrate through a hydrophilic matrix. Polar solvents such as acetone and tetrahydrofuran can be used for this purpose (12). More polar solvents are best for extraction of xanthophylls, while mixtures of hexane-acetone have been successful for extraction of total carotenoids. Recommended procedures involve first blending the tissue with water, followed by precipitation of carbohydrates and proteins with methanol-ethanol, which also serves to dehydrate the tissue. This allows for easy penetration and subsequent extraction of tissue with organic solvents. Homogenization of the tissue in the presence of
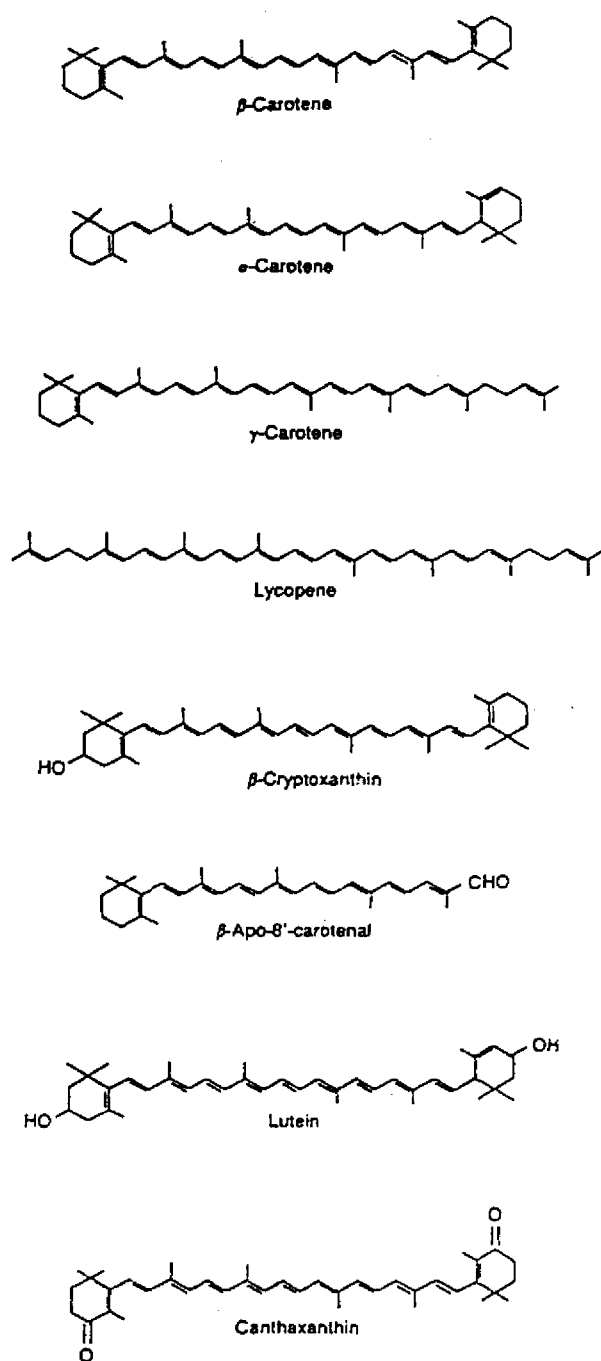
19-3
figure

Structures of prominent carotenoids.

matographic separation of carotenoids, saponification will rapidly degrade the chlorophyll constituents. Either hot or cold saponification procedures can be performed, generally with 40% methanolic KOH. However, controversy exists regarding whether or not xanthophylls and xanthophyll esters are completely hydrolyzed or partially lost during this procedure. Furthermore, heat treatments may cause isomerization reactions to occur, converting the all-*trans* from of the carotenoid to is *cis* geometrical configurations. If at all possible, saponification procedures should be avoided to alleviate the extra handling step and the possibility of pigment losses and artifact formation.

Numerous HPLC methods have been developed and optimized for the analysis of carotenoids in foods. Often the procedures are designed for the separation of specific carotenoids present in a particular fruit, vegetable, or food product (14). Both normal-and reversed-phase methods have been used for carotenoid analyses; however, reversed-phase methods have predominated. Commercially available C-18 columns have been popular, using both gradient and isocratic elution procedures. Nonaqueous mobile phases have been used successfully in conjunction with reversed-phase columns for the analysis of provitamin A carotenoids. Typical solvents employed include mixtures of methanol and acetonitrile containing ethyl acetate, chloroform, or tetrahydrofuran.

Detection wavelengths for monitoring carotenoids range from approximately 430 to 480 nm. The higher wavelengths usually are used for some xanthophylls as well as to prevent detection from interfering chlorophylls. Since the absorption maximum of β-carotene in hexane is 453 nm, many methods have detected the carotenoids near this region. When fixed wavelength detectors are available, 436 or 440 nm is popular but less sensitive. Detection sensitivity can be optimized by monitoring eluents at the absorption maxima of the carotenoid in question.

Often the identity of a particular carotenoid is found by coelution with a standard. It also is advisable to compare UV-Vis spectra of unknowns with authentic compounds. Standards can be purified on thin layer chromatography (TLC) plates using absorbants such as MgO_2, $Ca(OH)_2$, and diatomaceous earth. Many carotenoids exhibit spectral shifts upon reactions with various reagents, and these spectral changes are useful to assist in identification. Freshly prepared purified standards are recommended for instrument calibration since many carotenoids are labile and subject to decomposition. Further, the identity of the carotenoid can be confirmed by mass spectral and NMR data. However, this still excludes the determination of the structural configuration of optical isomers, which requires much more detailed analyses.

A typical chromatogram of the carotenoids present

the extracting solvents is preferred to denature proteins that may bind carotenoids and for complete quantitative extractions.

For samples high in fats and oils, saponification may be necessary to remove the lipids (13). Also, if chlorophylls are present and interfere with the chro-
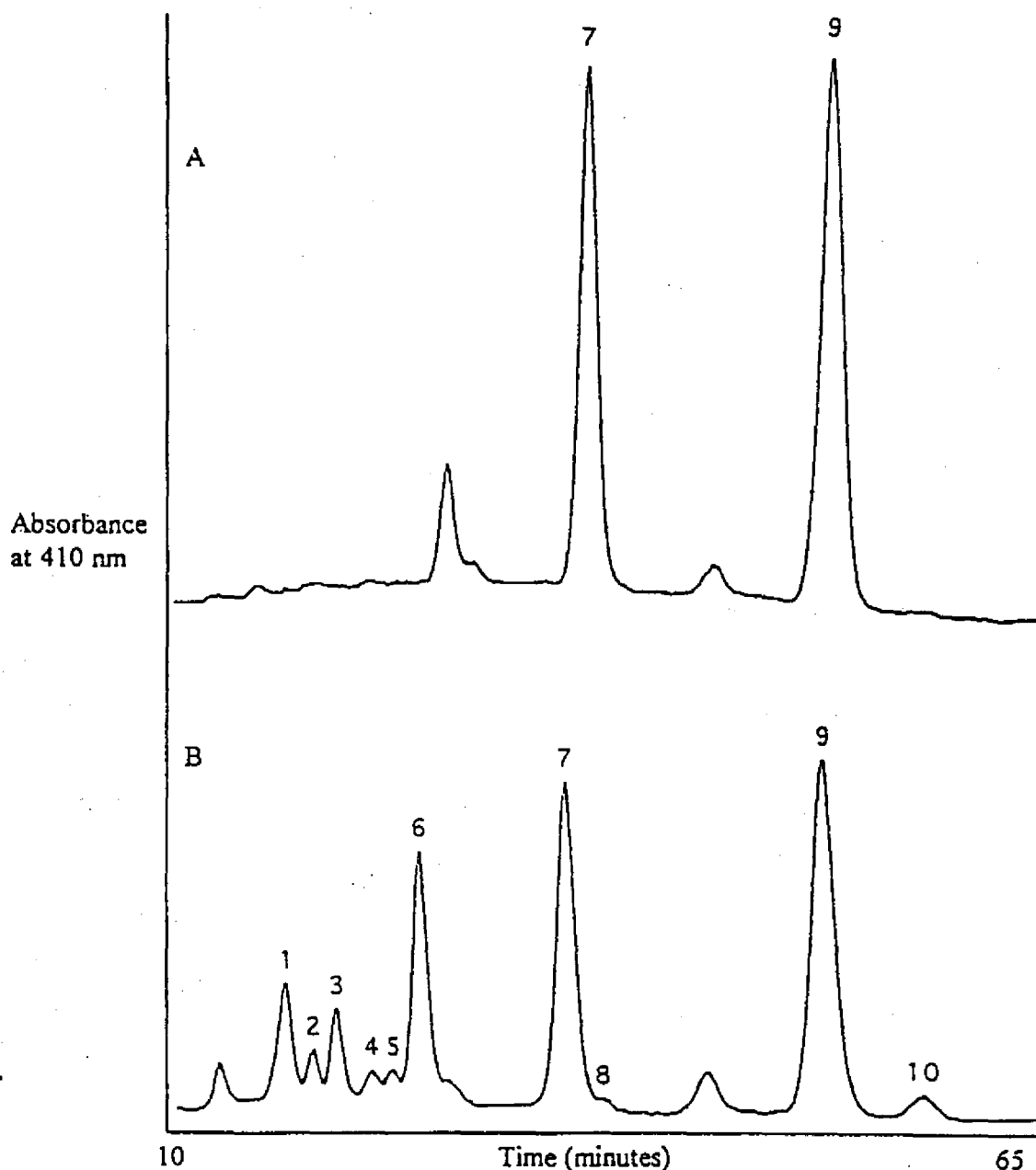
19-4
figure

Reversed phase HPLC separation of α-carotene (AC) and β-carotene (BC) isomers in (a) fresh and (b) canned carrots using a 5 μm C_{30} stationary phase. Peak 1 = 13-*cis* AC; 2 = unidentified *cis* AC; 3 = 13'-*cis* AC; 4= 15-*cis* BC; 5 = unidentified *cis* AC; 6 = 13-*cis* BC; 7 = all-*trans* AC; 8 = 9-*cis* AC; 9 = all-*trans* BC; 10 = 9-*cis* BC. [Reprinted with permission from (15). Copyright 1997 American Chemical Society.]

in a carrot extract is shown in Fig. 19-4A. The HPLC conditions used for the analysis are designed for the separation of provitamin A carotenoids (i.e., α, β, δ carotenes and cryptoxanthin). Most fruits and vegetables that possess provitamin A activity contain significant quantities of the mentioned carotenoids; however, the provitamin A activity varies considerably (16). Other methods are more appropriately designed for the analysis of the more polar xanthophylls. To resolve

and separate the xanthophylls, more polar solvent systems are often used. However, it is possible to resolve a complex mixture of xanthophylls in the presence of the less polar carotenes by using a selective solvent mixture or eluting the carotenoids with a gradient (17). Recently, a new C-30 stationary phase column has been specifically engineered for the analysis of pigments in plant tissues and is widely applicable toward the measurement of both polar and nonpolar carotenoids

(15,18), as well as *cis–trans* isomers. A typical separation is shown in Fig. 19-4B for a canned carrot sample. Further challenges are present and commonly encountered in fruits when carotenoid esters must be separated for analysis.

19.4 ANTHOCYANINS

The anthocyanins are important pigments responsible for the red, blue, and purple colors of some flowers, fruits, vegetables, juices, wines, and jams. Many different anthocyanins are found in commonly consumed plant products. A unique aspect of most anthocyanins is their ability to reversibly change color as a function of pH. The basic structure of the anthocyanins and the structural transformations that occur with pH changes are illustrated in Fig. 19-5.

The water solubility of anthocyanins provides for their relative ease of extraction from plant tissues. Timberlake and Bridle (20) have compiled an extensive list of anthocyanins found in commonly consumed plants. Many of these pigments are glycosides, commonly glucosylated, but other sugar moieties may be present along with a variety of esters. Methanol or ethanol containing 1% or less HCl is best for extraction of finely ground tissue. Lower concentrations of HCl (0.01–0.05%) may be necessary to prevent hydrolysis if anthocyanin glycosides are present.

Measurements of anthocyanins present in extracts, juices, or wines have been explained by Wrolstad et al. (19) and can be estimated by determining the absorbance of a diluted sample at pH 1.0 at the wavelength maximum (510–540 nm). The samples must be centrifuged if they are not clear. Concentrations can be estimated by using an appropriate known absorptivity value for a pure pigment solution. Selection of the absorptivity value is based upon the predominant anthocyanin present in the plant material and the

extracting solvent used. Interferences are subtracted out by using a pH differential method. Both pH 1.0 and pH 4.5 buffers are used to dilute the extracts. At pH 4.5, the anthocyanins are colorless and thus absorbance measurements attributed to only the anthocyanins are obtained by the difference. If any haze is apparent in the solutions, this can be corrected by absorbance readings at 700 nm as follows:

$$\text{Absorbance of anthocyanins} = (A_{\lambda max} \text{ pH } 1.0 - A_{700\,nm} \text{ pH } 1.0) - (A_{\lambda max} \text{ pH } 4.5 - A_{700\,nm} \text{pH } 4.5) \qquad [1]$$

Absorbance measurements for anthocyanin content provide estimates for total quantity; however, this determination does not account for the complexity and identity of the various anthocyanin pigments that are present in plant tissues. Early chromatographic methods utilized both paper and column chromatography to separate the various components. Paper chromatography is still popular for isolation and characterization in which milligram quantities of pigments are needed. Many modern HPLC methods now are available for this analysis. The water solubility of anthocyanins makes this class of compounds ideal for reversed phase methods employing C-18 columns. Solvent systems usually consist of a mixture of water; acetic, formic, or phosphoric acids; and either methanol or acetonitrile. Characterization of the anthocyanins has been enhanced with the use of photodiode array detectors for which full UV-Vis spectral information can be obtained as the compounds elute from the column (21) and applied toward the analysis of fruit and juice samples (22). More recently, the interest in characterizing these compounds has been renewed, because of the potential of these pigments to exhibit antioxidant activity. Thus, more detailed structural characterization have been performed using HPLC, gas chromatogra-

OXONIUM SALT   CARBINOL BASE   QUINOIDAL ANHYDRO BASE

(FLAVILIUM CATION)

pH 1   pH 4-5   pH 7-8

ORANGE-RED   COLORLESS   BLUE
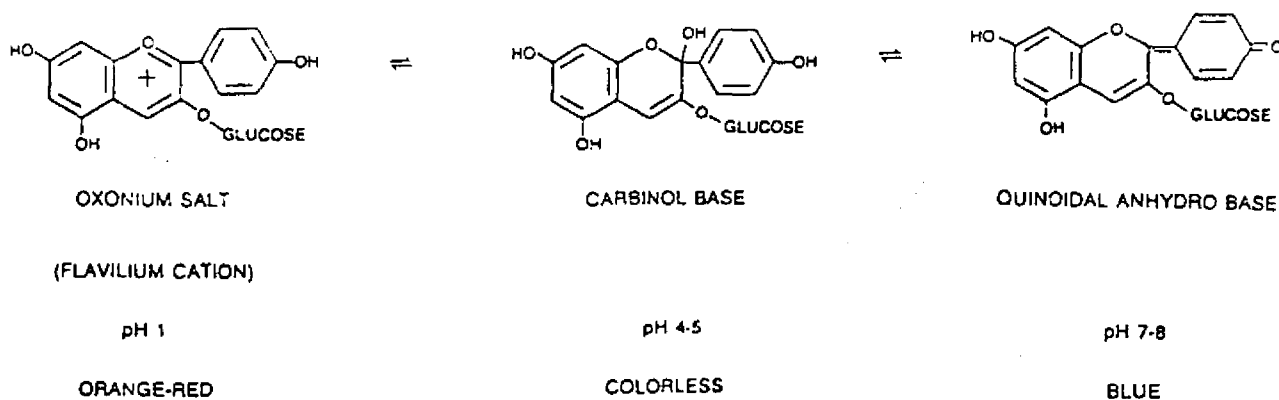
19-5
figure   Structural transformation of anthocyanins with change in pH. [From (39), used with permission.]

phy (GC), nuclear magnetic resonance (NMR), and mass spectrometry (MS) (23, 24).

19.5 BETALAINS

The betalain pigments are not widely distributed throughout the plant kingdom. The purple red beet root contains a high concentration of betalain pigments, which consist of the predominant purple-red betacyanins and lower concentration of the yellow betaxanthins. Figure 19-6 shows the structures of these compounds.

Betalain pigments are ionic, exhibit high water solubilities, and are therefore extracted readily from plant tissues with water (25). Initial homogenates are prepared by blending tissue (~100 g) with EtOH:H₂O (50:50, 100 ml). The ethanol is present to precipitate carbohydrate polymers and proteins and lessen any enzymatic reactions that might cause degradation of the pigments. Blended tissues are filtered with celite or filter aid and then are quantitatively washed with water until completely extracted.

The fact that the betalains consist of both the yellow betaxanthins and the red betacyanins precludes the use of direct spectophotometric measurements of either species. However, quantitative measurements can be performed prior to separation by accounting for the absorbances of each pigment as well as some impurities (26). The method is based on the fact that betanin and vulgaxanthin, the major betacyanin and betaxanthin, have maximum absorbance wavelength regions

BETANIN R = GLUCOSE

BETANIDINE R = H

PREBETANINE

R = GLUCOSE-6-SULFATE

VULGAXANTHIN I R = NH₂

VULGAXANTHIN II R = OH

Structures of betalains in red beets. [Reprinted from (25), p. 65, by courtesy of Marcel Dekker, Inc.]

at 535–540 nm and 476–478 nm, respectively. Second, betanin absorbs light at the absorption maximum of vulgaxanthin; however, vulgaxanthin does not interfere at the maximum absorption of betanin. Therefore, the ratios of the absorbances at 538 nm and 436 nm with a correction for impurities lead to a set of equations for the determination of the pigments. The equations Nilsson (26) developed were:

$$x = a - z \quad [2]$$

$$y = b - z - x/(A_{538}/A_{476}) \quad [3]$$

$$z = c - x/(A_{538}/A_{600}) \quad [4]$$

where:

a = the light absorbance values of the extract at 538 nm

b = the light absorbance values of the extract at 476 nm

c = the light absorbance values of the extract at 600 nm

x = the calculated absorbance contributions for betanin

y = the calculated absorbance contribution for vulgaxanthin

z = the calculated absorbance contribution for impurities

Using the known one percent solution ($E^{1\%}$) absorptivity values of 1,120 for betanin and 750 for vulgaxanthin, the concentrations of each can be calculated. Alternatively, a nonlinear curve-fitting procedure can be used to determine individual pigment contents from the mixture (27).

Because of the coexistence of the light-absorbing pigments present in extracts, chromatographic procedures have been developed to separate and analyze the individual components. The charged pigments can be separated by electrophoresis, but they are more rapidly resolved and analyzed by HPLC. Individual betacyanins can be separated on reversed-phase columns by using an ion-pairing or ion-suppression technique (28,29). These methods enhance resolution by minimizing complete ionization of the carboxylic acid groups present on the pigment structure. This allows for greater interaction of the molecule with the HPLC stationary phase and better separation of the ionic pigments, as shown in Fig. 19-7.

19.6 MYOGLOBINS

Myoglobins, responsible for the color of meats, usually are determined by reflectance spectrophotometry of the meat surface (30,31). These consist of three forms—metmyoglobin, oxymyoglobin, and deoxymyoglobin. The oxidative state of the iron located within the
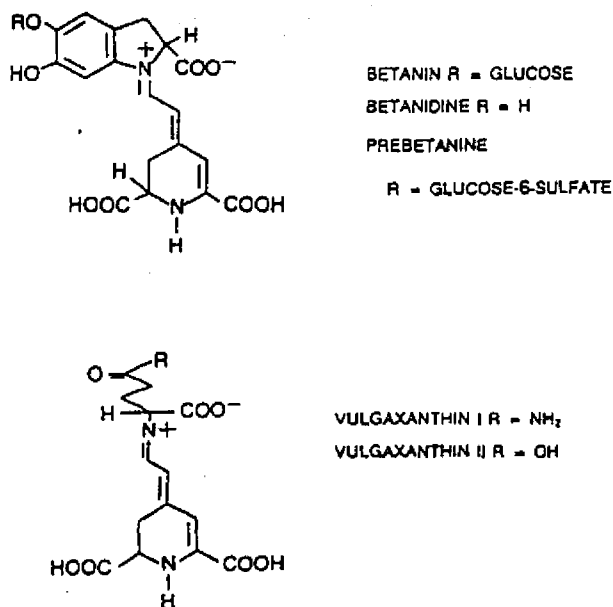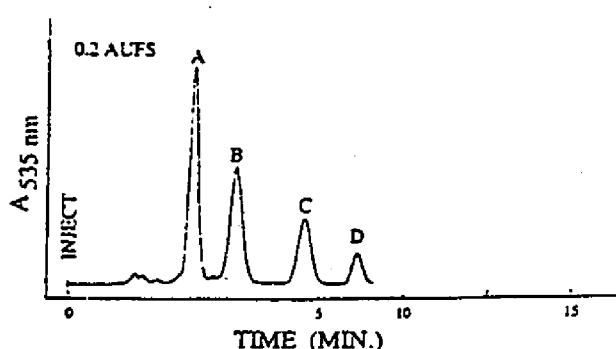
19-7 figure HPLC chromatogram of betacyanin pigments. A = betanin, B = isobetanin, C = betanidin, D = isobetanidin. [Reprinted from (25), p. 67, by courtesy of Marcel Dekker, Inc.]

porphyrin structure dictates whether the color is red (oxymyoglobin, Fe^{2+}) or brown (metmyoglobin, Fe^{3+}). Deoxymyoglobin (Fe^{2+})is purplish red in color but can be oxidized to the undesirable brown metmyoglobin. The chemistry of myoglobin systems has been reviewed by Faustman and Cassens (32).

The pigments present in meat also can be extracted and measured (33). Extraction is carried out by using phosphate buffer, pH 6.8, at an ionic strength of 0.04. The absorbance of the extract is due to a multicomponent system consisting of oxidized, oxygenated, and reduced pigment forms. By measuring the absorbances at 572, 565, 545, and 525 nm, the concentration of each pigment species can be calculated using derived equations (34).

19.7 SYNTHETIC FOOD DYES

There are seven synthetic food dyes approved for use in the United States under the Food, Drug, and Cosmetic (FD&C) Act of 1938, amended by the 1960 Color Additive Amendment (35). These dyes generally are much more stable to heat treatments, pH changes, and extended storage conditions relative to the natural colorants (36). For these reasons, they are used in small quantities in a variety of food products. Some ques-

19-1 table Certified Food Colorants

FD&C Color	Common Name
FD&C Red No. 40	Allura Red
FD&C Blue No. 1	Brilliant Blue
FD&C Blue No. 2	Indigotine
FD&C Green No. 3	Fast Green
FD&C Yellow No. 5	Tartrazine
FD&C Yellow No. 6	Sunset Yellow
FD&C Red No. 3	Erythrosine

tions about the safety of these dyes have been raised, but most concerns have been addressed in recent years by thorough studies conducted by the certified color manufacturers. The trivial names and FD&C numbers of these compounds are shown in Table 19-1.

Extraction of synthetic colorants from foods usually is not a difficult process for qualitative measurements. However, for quantitative measurements, complete removal of the colorants could be problematic because of an affinity of the dyes to bind to protein. Acidic solutions or various buffers may be used to extract the dyes. If acid dyes are present, ammonical alcohol is suitable as an extracting solvent. In the AOAC Method 930.38 (2) procedure, a liquid anion-exchange resin is employed to trap and purify the pigments from other food components. Digestion of the proteins and lipids with papain and lipase also has been proposed, but recoveries may not be completely quantitative.

Once extracted, identification and quantification of the pigment is obtained by measuring the UV-Vis spectrum. If more than one pigment is present, it is possible to derive a set of equations for the determination of the individual components. However, modern HPLC methods have been developed to rapidly separate and identify the dyes by coelution and comparison of spectra to readily available authentic standards. Most successful methods utilize an ion pairing technique that forms reversible ion pairs with the dye molecule anions (37). The ion pairs then are resolved on a C-18 reversed-phase column using polar eluent mixtures of water, methanol, and acid (38).

19.8 SUMMARY

This chapter has attempted to summarize some of the current methods for pigment analysis applicable to a food analysis course. Basic principles of extracting, handling, and storing pigments are emphasized since many naturally occurring pigments are relatively labile and susceptible to degradation. In addition, specific procedures for the analysis of plant and animal pigments including the chlorophylls, carotenoids, anthocyanins, betalains, and myoglobins are provided. Relatively little information on the analysis of synthetic dyes present in food products has been published. However, a brief section on the measurement of FD&C dyes is included for the reader's reference.

Although the advent of HPLC techniques with photodiode array detectors has markedly improved the analyst's ability to detect and quantitate food pigments, many complex analytical challenges exist. Further understanding of the biological state, chemistry, and interactions of pigments in food systems will be necessary to enhance our capabilities in extraction, separation, and detection techniques. In addition,

advances in instrumentation and rapid detection methods will improve and provide the food chemist with additional tools for rapid measurements of pigments and colorants in food products.

19.9 STUDY QUESTIONS

1. Given that chlorophyll and carotenoid pigments are both lipid soluble, what chromatographic assays (i.e., stationary phase and solvent composition) could be considered for their measurement in sample extracts? Consider specific procedures that measure for both classes of compounds in a single analysis.

2. Describe appropriate extraction methods for the lipid-soluble (chlorophylls, carotenoids) and water-soluble (anthocyanins, betalains, and FD&C dyes) pigments. For the water-soluble myoglobins, what changes in extraction procedures would be required?

3. Explain the limitations of the AOAC procedure for analysis of carotenoids in comparison to HPLC techniques. How do these limitations influence provitamin A measurements?

4. In high-fat-containing foods, lipids may interfere with the analysis of lipophilic pigments. Discuss what procedures can be used to minimize the interference.

5. Discuss why pH is an important factor when trying to analyze and separate anthocyanin pigments present in fruit juice extracts.

6. FD&C Red No. 40 is an example of a synthetic food dye. (a) What does FD&C stand for? (b) How do synthetic dyes differ in their properties from natural colorants? (c) What chromatographic method is most commonly used to separate and quantitate synthetic and natural colorants?

7. Describe the techniques used to measure the quantity of a single pigment present in an extract containing a mixture of pigments. Consider procedures that do and do not require chromatographic separation steps.

8. In the analysis of lipid-soluble carotenoid pigments in plant foods, why are polar solvents such as methanol and ethanol used within the extraction procedure?

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Analysis of Pesticide, Mycotoxin, and Drug Residues in Foods

William D. Marshall

20.1 PESTICIDE RESIDUES

20.1.1 Introduction

20.1.1.1 Regulations Governing Pesticides Residues in Foods

Pesticides continue to be used, on a large scale, to mitigate or limit the economic losses associated with decreases in crop yields (or quality) that are caused by noxious insects, fungi, weeds, or other pests. When applied improperly, residues of some of these pesticides can remain on foods and, as such, can pose a significant hazard to human health. Thus, in most countries, the sale, distribution, and ultimately the application and end use of these chemical and biological poisons are strictly controlled by law (1). To be offered for sale/distribution within the United States, a candidate control agent must be registered with the Environmental Protection Agency (EPA) (2). Other countries have adopted analogous procedures. The process of registration is an administrative procedure in which the agency reviews a detailed compilation of the chemical, biochemical, and environmental fate/behavior of the active agent and also extensively reviews the toxicology of the pesticide to both target and nontarget organisms. Included in this data package must be analytical methods for the determination of the terminal residues of the active ingredient on each crop that might be treated with the pesticide (and for more recent registrations, a requirement for information about the behavior of the active ingredient in standard multiresidue analytical methods also has been included). Thus, the process of registration involves an application to use the biological or chemical control agent at specified levels on specific crops.

The agency is charged with balancing the risks and benefits associated with the proposed use(s) of the candidate pesticide and to assure itself that neither humans nor the environment will be placed at undue risk. Registration status can be reviewed, altered, or revoked based on new toxicological, environmental, or residue data. In the latter case, the agency would issue a Rebuttable Presumption Against Registration (RPAR), which would be printed in the Federal Register and would outline the agency's reasons for the proposed revocation–alteration of registration and would provide interested parties a set time to respond and rebut the agency's arguments.

Based on the intended use of the candidate pesticide, EPA establishes a tolerance level, a legal limit of pesticide residue at harvest (which will include the active ingredient as well as toxic metabolites, and transformation products). Conventionally, tolerance levels are expressed in units of concentration and are in the range of low to sub mg/kg of fresh weight of edible produce [hence the term *ppm*, part per million (parts)]. These tolerances must be established prior to registration. Crop-specific tolerances cannot be legally exceeded, and residues are legally prohibited in or on food crops for which a tolerance has not been established. If experiments demonstrate that processing the raw agricultural commodity will concentrate residues of the control agent, a separate tolerance for processed products is issued.

Generic names (common names) for the active ingredients of products offered for sale have been developed by pesticide science societies so as to avoid reference to either their trade names or different formulations (which may be sold by different companies) containing the same active ingredient. Wherever possible, the International Union of Pure and Applied Chemistry (IUPAC) common name is used when more than one exists (which is frequently the case).

20.1.1.2 The Enforcement of Tolerances

Whereas registration in the United States is the prerogative of EPA, the responsibility for enforcing the tolerance limits is the responsibility of the Food and Drug Administration (FDA) which oversees all foods and feeds moving within interstate commerce, with the exception of meat and poultry products, which are the responsibility of the United States Department of Agriculture (USDA) (3). Included within this mandate are foods that have been imported into the United States. (See also Chapter 2.)

20.1.1.3 Changing Pesticide Usage Patterns

The vast majority of pesticides in current use are synthetic organic chemicals that are foreign to the environment (hence the term xenobiotic). This usage pattern can be expected to change slowly with time as accepted agricultural practice evolves from a chemical based to a combination of chemical and biological control practices. For example, the annual Western world market for microbial pesticides is anticipated to increase some 200-fold, to $8 billion, by the millennium (4). It also can be anticipated that the market share will continue to shift in favor of control agents that are less persistent, more selective in their biochemical mode of action, or can be used efficaciously at lower rates of application. In addition, integrated pest management (IMP) will become more efficient and more widely practiced. As the name implies, IMP is an approach to pest control that utilizes regular monitoring to determine if and when treatments are needed. IMP employs physical, mechanical, cultural, and biological tactics to maintain

pest numbers low enough to reduce economic losses to an acceptable level.

Pesticides are not biocides but rather are selectively toxic to target organisms. These chemical control agents have the ability to selectively disrupt specific biological processes. It is this high degree of selectivity that makes them useful as agrochemicals. Given the vast array of crop pathogens, crop predators, and other plants (weeds) that compete with the crop plants for limited nutrients, it is not surprising that no one control agent can protect even a single crop from all predators. Since these control agents selectively interfere with different biochemical processes, it is not surprising that they have different chemical structures and therefore very different physical and chemical properties.

Currently there are some 316 different pesticides (active ingredients) for which a crop specific maximum level of tolerable residue at harvest has been established (5). In addition, there are other pesticides: (1) with a pending or temporary tolerance; (2) for which no tolerance has been established, that have had a former registration status revoked, and that can be used in food production in other countries; or (3) for which metabolites, transformation products, or toxic impurities can be formed during the manufacture of the active ingredient. Table 20-1 provides a rough estimate of the numbers of chemical control agents within these different classes that collectively exceeds 740 different chemicals. Regardless of the exact total, it is small relative to the more than 8000 separate crop-specific tolerances that have been established. Given the large number of different possible residues, the different possible food types, and the level of advancement of current pesticide residue monitoring technologies, it is not

physically possible (or desirable) to test every food commodity for every pesticide residue.

20.1.1.4 Are Foods Safe?

Public concern for pesticide residues in foods has increased dramatically in the last decade (6). Increasingly articulated fears concerning food chemical safety have placed added pressures on government regulatory and monitoring agencies as well as on producers and processors to demonstrate that foods offered for sale are free from toxic chemical contaminants. These concerns are heightened by the possible deleterious health effect(s) of pesticide residues in the diet and a lack of information on the effect (if any) of continued exposure to combinations of pesticide residues. Finally, there is a sense that exposure to these residues is beyond the control of the consumer. The fact that currently it is not physically possible to test all foods for all possible residues is often interpreted as a lack of proof of the chemical safety of the food. Frequent surveys of our food supplies indicate, repeatedly, that the majority of the samples do not contain any detectable pesticide residues. For the years 1987–1995, the FDA has published (in the *Journal of the Association of Official Analytical Chemists / Journal of AOAC International*) a comprehensive annual summary of results of its pesticide monitoring programs in foods. The three most recent reports also are available on-line from http://vm. cfsan.fda.gov/~lrd/pestadd.html While not precipitous, each year the fraction of the total number of samples for which no pesticide residues were detected continues to increase. Samples in violation are typically <1% of the total numbers of samples.

20-1 table Numbers of Pesticides That Are Determined or Identified by the Principal FDA Multiresidue Methods[1]

Type of Pesticide	Total in Database	Total[2] for All Five Methods	Methods in PAM I[3]				
			4	5	6	7	8
Pesticide with:							
Tolerance	316	163	68	85	55	140	20
Temporary or pending tolerance	74	10	4	3	4	9	4
No EPA tolerance	56	25	17	21	7	10	0
Metabolites, impurities, etc.[9]	297	92	20	32	31	61	18

[1] Data as of May 1988 (reference 5)
[2] Entries in this column are not cumulative because more than one method can detect the same pesticide
[3] Standard multiresidue methods as outlined in *Pesticide Analytical Manual* (Volume 1) (PAM I).
[4] GC method for relatively nonpolar (organochlorine and organophosphate) pesticides in fatty foods
[5] GC method for relatively nonpolar (organochlorine and organophosphate) pesticides in nonfatty foods.
[6] GC method for organophosphonate pesticides and metabolites.
[7] GC method for polar and nonpolar pesticides using a variety of detectors
[8] HPLC method for N-methyl carbamates
[9] Only some of these pesticide-related compounds occur in foods or are of toxicological concern

We also must not lose sight of the concepts associated with relative risk. It has been estimated that we consume about 10,000 times more natural than synthetic pesticides (7). It seems probable that virtually every plant food that is available in supermarkets contains natural plant toxins, many of which are probable carcinogens. It has been suggested that "the carcinogenic hazard from current levels of pesticide residues or water pollution is likely to be minimal relative to the background levels of natural substances" (7).

20.1.2 Contemporary Analytical Techniques for Pesticide Residues in Foods

20.1.2.1 General Considerations

Given that the objectives of an analysis for residues can be quite different, the methodological approach must be matched to the problem. On the one hand, the problem might be to detect suspected residues of a particular active ingredient within a shipment of fresh produce that is known to have been field treated with the pesticide. Alternately, a considerably more complicated problem would be to determine the levels of residues of any pesticide(s) within that same shipment. As with all analytical procedures, the results are generated from a small quantity of an extract that actually is presented to the instrument. The results are of no use unless they can be used to make predictions about the levels of pesticide(s) that are present within the rest of the shipment. This can be done only if two conditions are met: (1) the analytical sample must be homogeneous and (2) the sample must be a miniature replica of the shipment itself. The problem of obtaining a sample that is truly representative of the levels of trace substances in a heterogeneous matrix is difficult to solve (see also Chapter 5). The problems associated with obtaining representative samples for the determination of trace contaminants in foods have been reviewed by Horwitz and Howard (8) and by Kratochvil and Peak (9). It is highly likely that the residues are unevenly distributed on exposed surfaces. Moreover, the food matrix itself is highly heterogeneous. For certain commodities, recommended sampling protocols have been established by both the AOAC International (10) and by the FDA (11).

Even when the samples are truly representative of the shipment, we often fail to recognize or to communicate to our customers that all of our analytical results are only estimates. The numbers we generate are not exact; rather they are subject to uncertainties. These uncertainties can be appreciable (12)—typically, ±10% if the analyte is present at the 1–10 mg/kg level and ±30 to ±60% if that same analyte is present at the 1–10 µg/kg level. It is not possible routinely to reduce these uncertainties appreciably.

20.1.2.2 Types of Analytical Methods for Pesticide Residues

There are several approaches to pesticide residue analysis. These methodological approaches vary in their degree of complexity; in the time, effort, and analytical instrumentation required to complete them; and in the degree of confidence that can be placed in the final results. Procedures may be quantitative multi-or single-residue methods, or only semiquantitative or even qualitative. Typically, one would use the least demanding procedure that will provide a level of confidence in the final results sufficient to answer the questions being posed.

20.1.2.2.1 Multiresidue Methods Multiresidue methods (MRMs) come closest to meeting the analytical needs of monitoring agencies charged with regulatory roles (13). MRMs have been designed to detect and measure a multiplicity of residues in a range of foods. They are sufficiently precise to provide reliable estimates of residue levels for many pesticides at or below the established tolerance levels. These multistep methods typically contain the steps of sample preparation, extraction, and cleanup, followed by chromatographic separation with on-line detection using a highly selective detector and automated quantitation. Of the 10 MRMs currently used by FDA and USDA, eight are based on gas chromatography (GC) and the remaining two on high performance liquid chromatography (HPLC). The MRMs that are used currently have evolved over a period of many years, yet they continue to be modified, expanded, and optimized. However, none of these MRM procedures can detect all residues on all crop types. In practice, they represent a compromise among the number of residues that can be detected, the range of food types that can be handled, and the levels of residues that can be measured. Their principal advantage resides in the numbers of different residues that they can detect and determine. A detailed description of the MRMs currently used by FDA is available in Volume I of the Pesticide Analytical Manual (PAM I) (11). A copy of this manual can be accessed electronically in a portable document format (PDF) via the internet at: http://vm.cfsan.fda.gov/~frf/pami3.html. AOAC International also has developed an MRM for pesticide residues, AOAC Pesticide Screen (AOAC Method 970.52).

20.1.2.2.2 Single-Residue Methods In contrast to MRMs, single-residue methods (SRMs) have been designed to measure a single analyte and, often, its principal metabolites and transformation products of toxicological importance. The majority of SRMs have been developed in support of applications for registration (including tolerance setting) or research into the

metabolism and environmental fate of the analyte. The majority of SRMs are based on the same sequential steps as MRMs; however, each step has been optimized for the analyte(s) of interest. Generally, SRMs are less time consuming to perform and often provide lower limits of detection than MRMs. However, they do vary in the level of validation to which they have been subjected. Volume II of the Pesticide Analytical Manual (PAM II) (11) consists solely of SRMs. Included in this volume are methods that have undergone EPA review (and possibly EPA laboratory evaluation) as well as certain methods that have been published in peer-reviewed scientific journals of high quality. In the latter case, these methods are similar to methods that have been approved by EPA but have been optimized for other commodities. In PAM II, those methods that have received EPA review are listed with roman numerals, whereas methods that have not been reviewed are lettered. At the time of writing, only an index of the contents of PAM II was available electronically (http://vm.cfsan.fda.gov/~frf/pam2.html).

20.1.2.2.3 Semiquantitative and Qualitative Methods

Semiquantitative and qualitative methods range widely in their abilities to estimate the level of a particular pesticide residue in a sample. In general, they are capable of detecting a limited number of somewhat similar pesticide residues. These methods are often referred to as screening methods in the sense that they are capable of assaying a large number of samples for the presence of a limited number of pesticide residues in a relatively short time. In addition, they are generally robust in character (they are less sensitive to small changes in the purity of reagents, quantities of reagents, time factors, reaction temperatures, or environmental conditions) and are not limited to a highly controlled lab environment. Whereas semiquantitative methods provide an estimate of the concentration range for a detected residue, qualitative methods will detect the pesticide if present above some predetermined level. The principal benefits of these methods are their low cost, relative speed, and simplicity. These methods make use of such techniques as thin-layer chromatography, enzyme inhibition, and immunoassay.

20.1.3 Quantitative Methods

20.1.3.1 Overview

The basic steps of a quantitative analytical method for pesticide residues include the following:

1. Sample preparation—The plant parts are separated into edible and nonedible fractions fol-

lowed by chopping, grinding, or macerating of the sample.

2. Extraction—Pesticide residues are removed from most of the sample's other constituents by solubilizing them in a suitable solvent. This step often involves blending the chopped sample with solvent in a homogenizer, followed by a filtration.

3. Cleanup (isolation)—The crude extract is purified further by removing those coextractives that can interfere in the subsequent determination step(s).

4. Separation—The components of the purified extract are further separated by a differential partitioning between a mobile phase (liquid or gas) and a stationary phase.

5. Detection and quantitation—A physical parameter of the separated components in the mobile phase is measured as they pass through a detector; this signal then is related to the quantity of analyte via a quantitation step.

There is a voluminous technical literature on pesticide residue analyses. Several methods have been used widely and have undergone extensive testing and validation for many different residues and different food types. There are several manuals (which are updated periodically) that provide detailed descriptions of the individual steps of these methods. Included among these compendia of methods are *Pesticide Analytical Manual* (Volumes I and II) (11); *Analytical Methods for Pesticide Residues in Foods* (14), published by the Health Protection Branch, Health and Welfare Canada; and *Analytical Methods for Residues of Pesticides* (15), published by the Government Publishing Office for the Ministry of Welfare, Health, and Cultural Affairs, of the Netherlands.

The choice of a method can be simplified somewhat by recognizing that the following criteria dictate which procedure(s) will be most efficient: the properties of the food matrix, the properties of the analyte(s), and the detectors that are available to the analyst. It is convenient to group the food types into one of four broad categories based on moisture and fat content: (1) high moisture, low fat (fruits and vegetables); (2) high moisture, high fat (meats); (3) low moisture, low fat (dried fruits); and (4) low moisture, high fat (cocoa beans). Although not exact, the high-low boundary is roughly 2% for fat and 75% for water. A detailed compilation of the moisture, fat, and sugar contents of a wide variety of fresh and processed foods has been compiled by Luke and Masumoto (16). The polarity, volatility, chemical reactivity, and thermal stability of the analyte(s) will determine the efficiency of recovery from the food matrix, the behavior on cleanup

columns, the choice of an appropriate analytical column, and the selection of an analytical instrument.

20.1.3.2 Sample Handling

Samples often arrive at the most inopportune times; often it is not possible to perform an analysis immediately. Sample handling procedures are designed to prevent any change of the sample in a way that would affect either the determination of the concentration of the analyte(s) or the nature of the analyte(s) (see also Chapter 5). If samples must be stored, it is essential that the conditions of storage be chosen so that the sample [both the analyte(s) and the matrix] deteriorates as little as possible. As with all biological materials, sample decomposition usually is retarded by storage in sealed containers at low temperature. Freezing the samples is recommended for certain protocols but otherwise avoided. Often it is preferable to perform part of the analysis and then to store a partially purified sample extract rather than store the whole laboratory sample itself.

20.1.3.3 Sample Preparation

Assuming that a laboratory sample has been provided that is a miniature replica of the food commodity for which residue data are required and that a working method that will provide the information required is available, the laboratory sample is prepared for analysis. The sample, as received, is divided into edible and nonedible portions; then a composite sample (often 1.5 kg) is prepared by chopping or grinding, followed by blending and mixing. A Hobart food chopper is very suitable for this purpose. Other food matrices can be handled best with a meat grinder, a hammer mill, or a larger-capacity food blender. These steps have two objectives: to reduce the structural features of the sample and facilitate the subsequent extraction, and to produce a homogeneous composite sample from which subsamples can be taken. Care should be taken to avoid contaminating the sample or exposing it to unnecessary heat, which can cause loss of volatile analyte(s) or accelerate decomposition.

Most pesticides are not systemic. They are not translocated within plants, nor do they traverse plant membranes. They can be expected to occur as surface residues on fresh produce and to be unequally distributed on those surfaces. Without special care, residues on the outer damaged leaves of leafy greens that typically are not eaten can easily contaminate inner leaves. Fresh fruits and vegetables as offered for sale are not washed prior to analysis (this has usually been performed by the producer), and only damaged outer leaves are removed. Pesticide tolerances have been established for raw agricultural commodities (RACs). Thus, the RAC is analyzed as received. If the outer skins are not usually consumed (onions, melons, or kiwi fruit), only the outer 2–3 mm are removed. Similarly, only stems of grapes and strawberries and the stems and cores of apples are removed. Although it would appear to be somewhat rare, the degradation of the surface residues of certain pesticides can be accelerated by the process of maceration with the food matrix. The degradation of the fungicide chlorothalonil on peas and captan and folpet on green beans can be accelerated by maceration. For field crops, when it is known that there has been no pretreatment, samples can be rubbed lightly to remove soil particles and other visible adhering contaminants.

20.1.3.4 Extraction

The objective of this stage in the analysis is to recover as much of the analytes as possible by solubilizing them. Often, a subsample (250 g or less) of the chopped or macerated composite sample is blended with a suitable organic solvent, generally acetonitrile (CH_3CN) or acetone ($CH_3C(O)CH_3$). Anhydrous salt (NaCl or Na_2SO_4) can be added to absorb water, or water can be intentionally added so that the crude extract can be purified with a subsequent partitioning step with a second water-immiscible solvent. The solvent is separated from insoluble solids by filtration.

A not infrequent problem at this stage of the analysis is the formation of an emulsion (a suspension of one solvent in a second immiscible solvent that masks the interface between them). Emulsion formation often can be minimized by adding a salt to the predominantly aqueous phase. Once formed, it can sometimes be broken down by centrifuging the mixture if feasible, or by adding a small quantity of saturated salt solution, a few drops of alcohol, or a commercial antifoaming agent. Less desirably, most emulsions will break down when left undisturbed for a few hours.

An alternate procedure (17) can be used to somewhat simplify the number of operations. Variations among different food types can be reduced appreciably by adding water to the sample to obtain a suspension that is ~70% water by weight. For samples that are >70% water (fruits, vegetables, wines, milk), a 100-g aliquot is taken. Dry samples (less than 40% water), 10–50 g, are presoaked (up to 2 hr) with sufficient added water to bring the water content to 100 g. For matrices that contain appreciable quantities of both water and fat (butter, animal tissues), sufficient water is added to the sample (10–30 g) to obtain a total of 100 g of water. Acetonitrile (200 ml) and dichloromethane (150 ml) or acetone (200 ml) and petroleum ether (150 ml) are added to the water-amended sample together

with sodium chloride (30 g). The mixture is blended at high speed for 1–2 min. The organic phase is dried over sodium sulfate, reduced in volume to 3–5 ml, diluted with 5 ml of an appropriate solvent, and reconcentrated. The dilution–reconcentration steps are repeated to ensure the complete removal of those extraction solvents that can disrupt the operation of the detector. The resulting concentrate can be used for analysis by gas chromatography without further purification unless an electron capture detector is to be used. For fruits and vegetables (100 g), the coextractives amount to a small fraction of 1 g. The more widely used procedures for extraction and cleanup have been reviewed by Walters (18).

20.1.3.5 Cleanup

20.1.3.5.1 Traditional Methods Often, the crude sample extract is partially purified before the separation/determination steps. The necessity for and the degree of cleanup required depend, to a large extent, on the instrumental detector to be used and to a lesser extent on the type of chromatography in the automated separation stage of the analysis. In general, it is the sample cleanup that is the most time-consuming, labor-intensive, and error-prone step of standard analytical methods. There is no universal cleanup procedure; instead, a variety of techniques have been used successfully. In this stage of the analysis, the analytes are separated from coextractives that can interfere with the detection of the analyte(s). Often, the preliminary partitioning step is followed by a preparative chromatography step. (See Chapter 31 for basic information on chromatography.) The crude water–acetone or water–acetonitrile extract is partitioned with a relatively nonpolar organic solvent. The organic phase is dried and reduced in volume. The residues then are further purified sometimes by a column chromatographic procedure (using either adsorption or size-exclusion chromatography). Typically, for adsorption chromatography, a 10–20 cm x 2.5 cm column packed with Florisil, silica gel, or less often alumina is used. The choice of packing material is both analyte and matrix dependent (Table 20-2). The activity of the adsorbent must be standardized (heated in an oven overnight, then deactivated by equilibrating with a prescribed quantity of water). It is sometimes convenient to make selective separations of the residues in the crude extract into groups based on their order of elution from the preparative column. Separate fractions of column eluate can be analyzed. The behavior of more than 200 pesticide residues on Florisil has been compiled in PAM I (11). Either isocratic elution or a stepped gradient of increasing solvent polarity can be used. Preparative size-exclusion chromatography represents an attractive alternative because it represents a

Stationary Phases Used for the Preparative-Chromatographic Cleanup of Pesticide Residues

[table 20-2]

Florisil® (usually 60–100 mesh)
1. A diatomaceous earth adsorbent that is well suited to the cleanup of nonpolar pesticides in fatty foods. Efficiently removes interferents when eluted with nonpolar solvents.
2. Less effective for the cleanup of more polar pesticides in fruits and vegetables.
3. Prone to batch-to-batch variations in activity.
4. Can accelerate the oxidation of certain organophosphate (OP) esters containing thioether linkages (thiolo compounds) and can adsorb, irreversibly, certain other OPs (oxons).
5. (R-S-R) can also be oxidized to sulfones (R-S(O)-R) and to sulfones (R-S(O)(O)-R) and strongly retained by this material.

Silica gel (a highly gelatinous form of silica)
1. Useful for the isolation of more polar pesticides and for the partial cleanup of nonpolar pesticides (organochlorines, OCls) from animal fats.
2. Will not adequately separate plant coextractives from certain pesticides.

Alumina
1. This material tends to be more alkaline and can decompose certain OPs.
2. Can be substituted for Florisil in the cleanup of fatty foods.
3. Does not adequately separate plant coextracts from certain pesticides.

Carbon
1. Preferentially absorbs nonpolar and high-molecular-weight pesticides.
2. Efficiently removes chlorophylls but not waxes from vegetable extracts.
3. Pretreatment of this material strongly affects its adsorption behavior. Flow rates with open tubular columns are difficult to maintain.

Size-exclusion packings
1. These materials separate mixtures principally according to size (not exclusively). High-molecular-weight materials are eluted first.
2. They must be preequilibrated (swollen) with the solvent or solvent mixture.
3. Losses of analyte residues are minimal (less than 10%) and are effective for the separation of animal fats and plant waxes from nonpolar pesticides.
4. Procedures often couple size exclusion with a short alumina column to remove residual carotenoids

different chromatographic mode than adsorption, it provides excellent recoveries (usually >80%), and it can be automated readily. Typically, the extract is added to 50–60 g of 200 to 400 mesh Biobeads SX3 (that have been preswollen by equilibration with the mobile phase), and eluted with methylene chloride or with acetone–cyclohexane mixtures (typically 25:75, vol/vol).

20.1.3.5.2 Alternative Techniques Increasingly, alternative techniques for sample extraction and purification are being adapted for pesticide residue analysis. The principal advantages of these alternative approaches are the decreased time required to complete the operation and the decreased quantities of solvents consumed. The latter savings can be appreciable when the cost to dispose of solvent wastes is considered.

1. Solid-Phase Extraction. Solid-phase extraction (SPE) typically involves small quantities of dry 30–40 μm diameter packing material contained in a single-use polypropylene or glass cartridge (30 mg–10 g of sorbent) or disk that can be used to purify, concentrate, or less frequently to derivatize the analyte(s) prior to chromatography/quantitation (see also Chapter 33, section 33.2.2.4). For cartridge materials, typical retention capacities are ~5% of the sorbent mass. However, one must account also for the other components of the crude extract that can be retained on the sorbent material. Minimum elution volumes are only 120 μl per 100 mg of packing material. Although most packing materials are silica based, polymeric packings also can be used. Many of the common liquid chromatography packing materials are available in one or more of the SPE formats as well. Given the limited resolving power of these systems, conventional strategies are to either selectively retain the analyte(s) on the packing material (during the loading and rinsing cycles) or conversely retain interfering impurities while selectively eluting the analytes from the packing material (elution cycle). An intermediate wash/rinse cycle can serve to separate other contaminants from the adsorbed analytes. Despite the relatively recent commercialization of cartridges/disks, manufacturers/distributors have built up an extensive collection of applications bibliographies (19–21). An alternative format replaces the cartridge with a 25, 47, or 90 mm diameter disk contained within a holder. SPE disks consist of sorbent particles (~5 μm diameter) typically either bonded-phase silica or polymeric material contained within a mesh of an inert porous support material such as microfibers of Teflon³. To minimize clogging of suspended solids to the surface pores of the disk, a bed (up to 1 cm thick) of a filter aid such as nonporous glass beads (~40 μm diameter) sometimes is used.

2. Solid-Phase Microextraction. A related technique, solid-phase microextraction (SPME), involves immersion of a polymer-coated fiber within an aqueous extract or retained within the headspace of the sample to enable the analyte(s) to diffuse into the coating. After equilibration, the fiber/needle assembly is transferred to the injector port of a GC where the analytes are thermally desorbed onto the column. Modules to adapt existing gas chromatographs to perform this technique are available commercially.

3. Microwave-Assisted Solvent Extraction. Mi-

crowave-assisted solvent extraction generally can decrease appreciably the extraction time and the quantity of solvent required to efficiently recover target analytes from the food matrix. Commercial systems are available that incorporate the capacity for simultaneously extracting multiple samples within closed, lined (perfluoroalkoxy) pressurized vessels (up to 8–12) that can be fitted with a fiber optic probe to accurately monitor and control the temperature within the vessel. The extraction vessels are fitted with a rupture membrane that will fail in the event of an over-pressure situation and one of the vessels is fitted with a pressure-sensing side arm that will turn off the microwave energy if the pressure exceeds a set amount. As an additional safety feature, a solvent detection device will turn off the unit in the event that solvent vapors escape into the cavity. The advantages of this approach include operation at temperatures that exceed the boiling point of the solvent(s), more rapid transfer of the analyte(s) to the solvent phase, and programmable operation. After extraction, the vessels must be cooled to ambient temperature, and the solvent separated from the sample matrix. Like microwave-assisted solvent extraction, a related technique, an automated Soxhlet system, can be used to automate and accelerate the extraction process.

4. Accelerated Solvent Extraction. The accelerated solvent extraction technique involves the use of limited quantities of conventional solvents at elevated temperature (up to 200°C) and/or pressure (1500–2000 psi) to statically extract solid samples for short periods of time (often <10 min) (see also Chapter 13, section 13.3.1.6.2). The approach is similar to microwave-assisted extraction but with the microwaves replaced by conventional heating. During heating, excess pressure is avoided by venting the solvent into a collection vial. At the termination of the extraction program, the remaining solvent is transferred to the collection vial with the aid of a compressed gas. Other automated units can be used to separate fats and higher molecular weight lipid materials from pesticide residues by a process of size exclusion.

20.1.3.6 Derivatization

It is sometimes advantageous to alter the chemical structure of analyte residues to make them more suitable for detection by chromatographic techniques. This process of structural modification by chemical reaction, referred to as derivatization, can be used to enhance the thermal stability or volatility of the analyte(s), to modify chromatographic behavior, to increase the selectivity of the detection, or to increase the sensitivity of detection (see also Chapter 33). Most often, derivatization is used to overcome limitations of sensitivity. Typically, substitution or addition reactions

are used to introduce a chromaphore, fluorophore, or other functional group into the analyte(s) to augment the detector response to the resulting product(s). Typically, the derivatization reaction is performed pre-column but post-column reactions also can be used provided that the reaction can be performed in situ and automated. The one requirement is that the identity of the original analyte(s) not be compromised by the reaction. The advantages of the derivatization must outweigh the disadvantages of increased sample handling, analysis time, and typically a decrease in precision. A wide variety of derivatization reactions have been used for selected pesticide residues (22, 23). Not surprisingly, this approach is limited to either single-residue methods or methods that determine a limited group of structurally similar pesticide residues.

20.1.3.7 Automated
Chromatographic Separation

20.1.3.7.1 Gas Chromatography (GC) GC has been used routinely for pesticide residue determinations for some 35 years; it is the instrumental approach of choice for most analysts. (See Chapter 33 for general information on gas chromatography.) An extensive technical literature on the behavior of residues with this technique has been built up.

For the vast majority of pesticide residue problems, it is enormously advantageous to follow a standard method. To choose a stationary phase for a custom analysis or for method development studies, a simple rule of thumb is helpful: like dissolves like (nonpolar analytes will interact most strongly with nonpolar liquid phases and vice versa). Analytes will behave quite differently on different liquid phases even if all other analytical conditions are identical. An extensive compilation of the retention times of many pesticides relative to an internal standard (chlorpyrifos, which contains the heteroatoms P, S, N, and Cl in addition to C, H, and O) on OV-17, on OV-101, and on DEGS has been presented by Froberg and Doose (24). These authors also provide many helpful suggestions on packed column preparation, conditioning, care, and rejuvenating. With time, there will be a gradual but inevitable buildup of sample coextractives at the head of the packed column (signaled by peak tailing, reduced response or retention times, or even analyte degradation). To restore performance, the initial 4–8 cm of packing can be removed, the exposed glass cleaned, and the packing replaced with fresh preconditioned stationary phase. Relative to other stationary phases, the DEGS material has a greater tendency to bleed (slowly volatilize), which can foul detectors.

Capillary columns, a second format of GC columns, are fabricated from fused silica and are jacketed with an impervious polyamide coating. Their principal advantage is their increased resolving power (roughly 12-fold over packed columns of similar lengths), which is achieved at the expense of sample capacity. In addition to providing increased resolution, the increased chromatographic efficiency of these columns (sharper peaks) results in better limits of detection. The use of capillary columns for routine pesticide residue analysis is commonplace despite the possibility of fouling problems due to coextractives.

Columns intermediary between packed and true capillary (0.75–1.0 mm internal diameter), megabore columns, also are available and present many of the best characteristics of both formats. Older chromatographs need not be modified; megabore columns retain much of the resolving power of true capillary columns even at higher flow rates, yet they retain sample capacities that are midway between packed and capillary columns.

The successful application of GC to pesticide residue analysis is critically dependent on the use of sensitive and highly selective detectors. The great majority of pesticides contain one or more heteroatoms (atoms other than H, C, or O) within their molecular framework. The presence of heteroatoms is exploited advantageously by using detectors that provide a greatly enhanced response to these heteroatoms. These include flame photometric (for S or P detection), electron capture (halogens, S and N), electrolytic conductivity (halogens or N and S), and thermionic detectors (N or P). With the exceptions of atomic emission spectroscopy (Chapter 28) and mass spectrometry (Chapter 29), other detectors have been applied to pesticide residue analysis only infrequently because of a lack of sensitivity.

Mass spectrometry (MS) (see Chapter 29) offers enormous possibilities as a highly selective means of detection and quantitation of pesticide residues. This technique offers unparalleled performance in terms of selectivity, in confirmatory power, and in the universality of analytes that can be detected. Dedicated systems for gas chromatography (mass selective and ion trap types) continue to decrease in cost and to improve in performance. These devices provide maximum response when only a few masses are monitored selectively [selected ion monitoring (SIM), as opposed to recording complete mass spectra]. For multiresidue monitoring, there is a requirement that the mass detector be capable of rapidly switching between preselected masses as the chromatogram develops. Continued improvements in recent years have been made in this area.

Components of the mixture subjected to GC analysis are identified, tentatively, based solely on their retention times (i.e., the retention time of the component is identical to the retention time of an authentic standard that has been chromatographed under identi-

cal conditions). Frequently, the retention time for an analyte is expressed relative to the retention time for an internal standard that has been added intentionally to the sample. The pesticide chlorpyrifos has been used often as an internal standard because it chromatographs well on many columns and is detected by all selective GC detectors.

20.1.3.7.2 High Performance Liquid Chromatography (HPLC)

The application of HPLC to pesticide multiresidue analysis has been restricted largely to those analytes that do not possess either the volatility or the thermal stability required for GC. Typical among these analytes are N-methyl carbamates [R-C(O)NH-CH$_3$], which are decomposed thermally at normal GC operating temperatures. (See Chapter 32 for general information concerning HPLC.) One advantage of the HPLC approach to pesticide residue analysis is that sample cleanup is usually less extensive. An extensive compendium of references for the analysis of pesticides by HPLC has been compiled by Muszkat and Aharonson (25); other reviews by Lawrence (26) and by Moye (27) also provide an overview of the application of the technique to pesticide residues.

The two detection techniques that have been most popular for HPLC determination of pesticide residues are ultraviolet (UV) and fluorescence spectrometry (see Chapters 26 and 32). Fluorophores (fluorescent tags) can readily be added to reactive functional groups of those analytes that are not naturally fluorescent. By contrast, the addition of chromaphores to reactive functional groups of target analytes typically does not provide sufficiently enhanced sensitivities to by useful. This process of derivatization, which increases the number of analytes or the response to these analytes, can be performed either pre- or post-column.

20.1.3.8 Quantitation

20.1.3.8.1 Overview
Since some physical parameter of the sample subjected to GC or HPLC is actually measured by the detector (ability to capture electrons, or to absorb light, etc.), the analog signal from the detector must be related to the quantity of analyte via a separate process of calibration. It is assumed that the change in the detector signal as the component analyte passes through the detector is caused only by that component. A recording device provides a record of the changes in the detector signal with time (a chromatogram). The result is a series of approximately Gaussian peaks corresponding to the separated components of the mixture. The time (or volume of mobile phase) required to reach the maximum of a particular peak is referred to as its retention time, and the peak height (vertical displacement from the base line) or peak area is used to

determine the quantity of analyte that must have caused that displacement. Components of the mixture are identified, tentatively, based solely on their retention times (i.e., the retention time of the component is identical to the retention time of an authentic standard that has been chromatographed under identical conditions).

Frequently, the retention time for an analyte is expressed relative to the retention time for an internal standard that has been added intentionally to the sample. The pesticide chlorpyrifos has been used often as an internal standard because it chromatographs well on many columns and is detected by all selective GC detectors.

Quantitation is performed, typically, by a method of external standards. A series of standard solutions is prepared by dissolving known quantities of authentic standard pesticide in a suitable solvent. These standard solutions are separately chromatographed to establish the response of the detector (in terms of either peak area or peak height) for the quantity of analyte injected into the instrument. A relationship between response and quantity of analyte then is established by regression analysis (the former on the latter).

Since the GC and HPLC methods just described are designed to determine multiple pesticide residues, they represent a compromise in terms of the quantity of each analyte that is actually isolated or recovered by the procedure. These procedures, although efficient, are not quantitative for most pesticide residues. Moreover, the recoveries themselves can be somewhat crop dependent. Data concerning the recoveries of residues using MRMs published in FDA's PAM-1 and their behavior in PAM-1 GC systems is available from: *http:/vm.cfsan.gov/~frf/pestdata.html*

20.1.3.8.2 Ancillary Devices For Automated Chromatography
It was inferred earlier that the major source of variation and uncertainty in most residue methods resulted from the cleanup step, which is laborious and time consuming as well as error prone. This is especially true if ancillary devices are used to increase the degree of automation of the separation and detection/quantitation steps of the analysis. Such ancillarly devices to automate chromatography include recording integrators, chromatography software packages, and autoinjectors.

A recording integrator will not only determine the retention time of each component of the mixture, but also will determine the peak area and/or peak height of each component as well as the corresponding area/height ratio. These robust, low-cost dedicated units make the successful outcome of the determination somewhat less critically dependent on the volume of sample injected into the chromatograph. The determination of peak height or peak area is achieved using

sophisticated mathematical algorithms that provide more precise and more accurate measurements of these parameters than is available using a strip chart recorder. Moreover, these measurements are independent of the visual presentation of the chromatogram. Thus, accurate quantitation can be achieved even if attenuation of the detector signal results in very small peaks for some trace components of the mixture. Since real chromatograms rarely contain truly Gaussian peaks, operator-selected variables (peak width, threshold, and area reject) are optimized to achieve reliable results.

Microcomputer-based chromatography software packages can increase the ease of the quantitation process. Retention times of peaks that have been detected can be calculated relative to an internal standard; then the resulting relative retention times can be compared with a database for standards to assign a probable identity. In addition, two or more chromatograms (or regions of chromatograms) can be visualized on the screen for comparison. Typical applications might include the comparison of two chromatograms generated from the same extract with different selective detectors or the comparison of a test chromatogram with a control (pesticide-free) chromatogram of the same food matrix. A review by Stan (28) provides a concise overview of the capabilities of personal-computer-based software for the evaluation of pesticide residue chromatographic data.

An autoinjector can be used to deliver a preset volume of sample to the chromatograph. This increase in automation frees the operator for other tasks (and, in theory at least, permits 24 hr operation) but, more importantly, it increases the precision associated with the injection step. Other automation can involve the use of column switching valves so that samples can be directed to different columns.

20.1.3.9 Chemical Confirmation of Pesticide Residues

The degree of confidence that can be placed in a peak assignment may not be sufficiently high; it is often preferable to corroborate the identity of the residue that has been detected. It is generally agreed that the most reliable way to increase the level of confidence concerning the identity and amount of an analyte present in a sample is to obtain concordant results using two independent methods that are based on entirely different analytical principles. This is not always feasible—multiresidue methods not based on a chromatographic separation are simply unavailable. For pesticide residues, it is customary to make use of one or more of the following less than ideal approaches. GC-MS can be used to (1) record the mass spectrum of the analyte and (2) provide a separate estimate (quantita-

tion) of residue levels. Alternately, a different selective detector can be used to replace the one used for the original analysis, or a second column with an appreciably different stationary phase can be employed. Finally, the analyte can be chemically altered, and then rechromatographed to demonstrate that the signal for the analyte has disappeared and has been replaced by a second signal at the predicted retention time for the anticipated transformation product. Two reviews (20, 29) explore these approaches in depth.

20.1.3.10 Immunoassays

Immunoassays are a group of related analytical techniques whose basis of commonality is the use of antibodies that have a high affinity for, and only for, the pesticide analyte. Immunoreactions are highly selective (virtually specific) addition reactions between the antibody (a high-molecular-weight glycoprotein that exhibits the properties of immunoglobulins) and the analyte of interest. This interaction can be exploited analytically if there is a means of detecting and, preferably, of quantifying the reaction, typically by competitive inhibition or by displacement in which the binding of the pesticide to the antibody competes with or displaces a tracer molecule. (See Chapter 21 for a detailed description of immunoassay techniques.)

The merits of immunoassays include their high selectivities and the simplicity, speed, and moderate cost of the procedure relative to other methods for pesticide quantitation. The principal disadvantage of the immunochemical approach is the extensive effort (and time) required to elicit the antibodies in a vertebrate host. A major concern of immunoassays is the degree of crossreactivity (affinity) that the antibodies show for related chemical structures. Thus, antibodies that were developed to one pesticide may react with other related chemical structures. Usually, this cross-reactivity is characterized by a lower affinity for the related structures than for the analyte itself.

A complicating factor for pesticide residue analysis is the nonpolar nature of the parent compounds that can be only sparingly soluble in the aqueous buffers commonly used in immunoassays. Providing that a monolayer of water can be maintained around the bound antibodies, assays sometimes can be performed in nonaqueous media. The kinetics of the process can be modified appreciably by the change of solvent. For certain assays, such as for carbendazim, successful analyses can be performed on crude ethyl acetate extracts without cleanup. By contrast, other procedures (for polychlorinated biphenyls) are successful only with a cleanup as extensive as required for GC. Recent reviews (30–32) on the application of immunoassays to pesticide residues are illustrative of the current status and the potential of this approach.

20.1.4 Thin-Layer Chromatography as a Semiquantitative and Qualitative Method for Pesticide Residues

Relative to automated chromatographic techniques, thin-layer chromatography (TLC) generally provides roughly one tenth of the resolving capabilities of a packed column for GC. In addition, the reduced precision associated with quantitation (relative to either GC or HPLC) and the limits of detection have caused this approach to fall somewhat into disfavor. (Refer to Chapter 31 for a discussion of TLC.) However, TLC has certain features that can be exploited advantageously, particularly when used as a semiquantitative screen for a limited group of pesticide residues. Quantitation need not be performed on-line; thus, a wide variety of static visualization techniques have been developed. In addition to appreciable improvements in resolving power that can be obtained with a smaller particle size stationary phase (high performance thin-layer chromatography, HPTLC), devices for reproducibly spotting the sample onto the plate and for quantitation by in situ densitometry appreciably improve the reproducibility of the technique. Extensive reviews on the detection and determination of pesticide residues by TLC are available (33, 34).

One successful application of TLC has been the detection and estimation of pesticides that inhibit cholinesterases. Many organophosphate (OP) and carbamate insecticides are capable of inhibiting this group of enzymes. A crude extract containing the suspect residues is separated by TLC. The developed plate is dried and sprayed with a solution containing one (or more) of these enzymes (often a partially purified tissue extract) and then with a substrate that, when hydrolyzed by the enzyme, liberates a colored product. The lack of color development indicates enzyme inhibition, so that the pesticide residues are visualized as colorless areas within a colored background. The zone of inhibition is proportional to the quantity of inhibitor present. However, many OPs (thiophosphate and dithiophosphate esters) must be activated by oxidation to their oxon analogs to serve as potent inhibitors. The stability of oxons is such that it is often preferable to perform the oxidation after the plate has been developed. Care must be taken that traces of oxidant do not interfere with the subsequent color development. More importantly, there are a number of other inhibitors of cholinesterases that occur naturally in foods. A kit based on cholinesterase inhibition has been developed by Enzy Tech Inc. (Kansas City, MO). It is claimed to detect, at low ng/g levels, all cholinesterase-inhibiting insecticides (35). Neogen Corporation (Lansing, MI) (http://www.neogen.com) also distributes a detection kit to monitor pesticide residues including malathion, carbaryl (sevin), carbofuran, sulfallate (vapona), metasystox, guthion, dursban, and diazenon.

A somewhat similar approach is the chromogenic detection of photosynthesis-inhibiting herbicides. Phenylureas, phenylcarbamates, 13 uracils, acylanilides, and triazines can be detected on TLC separations of crude extracts that have been partitioned into dichloromethane (36). The eluted plate is sprayed with a suspension of chloroplasts, then with a redox indicator (2,6-dichloroindophenol) and exposed to sunlight. Photosynthetic inhibitors are visible as blue spots of unreacted dye.

20.1.5 Example Procedures

Now that various aspects of analytical techniques for pesticide residues in foods have been covered, short summaries of two current MRM procedures to determine pesticide residues are outlined below. For the AOAC Pesticide Screen (AOAC Method 970.52), a ground, homogeneous sample is extracted with acetonitrile. The extract is diluted with water and the extracted compounds are partitioned between petroleum ether and acetonitrile. The crude extract from the partitioning step is purified by eluting from a Florisil or magnesia column with petroleum and ethyl ethers. The eluate is concentrated and analyzed by gas chromatography with selective detection. Minimum detection levels are in the range of 0.01 ppm depending on the sample matrix. A second procedure is directed to the determination of N-methylcarbamate pesticides by HPLC. The EPA Method 531.1 for foods and waters is based on a reversed-phase separation of the carbamate analytes with on-line post-column derivatization by base/o-phthalaldehyde followed by fluoresence detection. For water, a binary gradient from water to methanol is used; limits of detection are sub µg/kg.

20.2 MYCOTOXIN RESIDUES

20.2.1 Introduction

Mycotoxins are a broad group of chemically diverse toxins (natural products that are toxic to some other organism) that are produced as secondary metabolites, principally by the terrestrial filamentous fungi commonly known as molds. The filamentous nature of molds is ideally suited to growth over surfaces and through solid substrates; the mycelium forms an efficient system to utilize the available nutrients and transport them to the growing hyphal tips. A consequence is that secondary metabolic products, including mycotoxins, will remain localized and highly concentrated in the food. The description secondary metabolites suggests only that these chemicals are not required by the producing organism for growth.

Mycotoxins can be produced as a result of fungal infection either in the field or in storage. In addition,

there is not a good correlation between the level of fungal infection and the levels of the mycotoxin(s) in the contaminated produce. Failure to detect viable inocula of a particular toxigenic species in fresh or stored produce is not a certain indicator that the mycotoxin is absent nor does the presence of that species guarantee that it will have produced toxin(s). Thus, features of this class of toxins are that individual members are frequently produced only by a specific species, and levels of production vary greatly not only among different strains of that species but also in response to environmental and nutritional conditions. For example, particular strains of *Aspergillus flavus* are used in the manufacture of koji, whereas other strains can produce aflatoxins. Numerous studies have demonstrated that several different mycotoxins can be present in the same sample.

Analytical methods for the detection and quantitation of mycotoxin residues in foods and feeds are necessary to ensure that these commodities are safe for human and animal consumption. Although the toxic effects vary greatly among different members of this class, they are generally relatively small molecules (MW <1000) and, typically, are not in themselves antigenic. They do not appear to accumulate in the body, and their toxicological effects, which can be acute but rarely fatal, vary widely. On the other hand, aflatoxin B_1 has been reported to be the most potent naturally occurring carcinogenic substance known. Despite this fact, the principal concern, at least for humans, remains the deleterious health effects associated with chronic exposure.

Although between 300 and 400 mycotoxins are known, the following order of relative importance for the common mycotoxins has been suggested (37): aflatoxins (hepatotoxins), ochratoxin (nephrotoxin), trichothecenes (dermatoxins), zearalenone (estrogen), deoxynivalenol (dermatoxin), fumonisin (immunotoxin), and citrinin (nephrotoxin). To limit human exposure to mycotoxins, recommended advisory levels (typically 5–20 µg/kg for aflatoxins) in foods and animal feeds have been established in many countries (38). As with pesticide residues, a number of analytical methods for screening, survey, and regulatory control have been developed and validated by interlaboratory collaborative studies. Organizations such as the AOAC International, American Oil Chemists' Society (AOCS), American Association of Cereal Chemists (AACC), and the IUPAC have mycotoxin method validation programs.

20.2.2 Sampling

For mycotoxins, the problem of obtaining a sample of the produce that is representative with respect to burdens of mycotoxins in the shipment is especially diffi-

cult. (See Chapter 5 for a general discussion of sampling.) It is very informative to consider the findings of a study (39) in which the levels of aflatoxin were determined in twenty 2.27-kg samples from each of 10 contaminated lots of cottonseed (Table 20-3). Each 2.27-kg sample was comminuted in a sampling mill and a 100-g subsample then was removed and analyzed following a standard method (extracted and the levels of aflatoxin determined densitometrically after separation on a minicolumn). In Table 20-3, the 20 analytical results for each of the 10 lots are ranked from low to high to facilitate comparisons. Rather than being symmetrically distributed about the mean (the "best" estimate of the aflatoxin concentration in the lot), the distribution of test results, for each of the 10 lots, is positively skewed (there are more values below the mean than there are above the mean). If a single 2.27-kg sample had been tested, there would have more than a 50% chance that the result would have been less than the true lot concentration. In general, the degree of skewness is greatest for small sample sizes and decreases as the size of the sample is increased. However, it can also be seen from the results of Table 20-3 that, even for the same sample size (2.27 kg), as the level of contaminant increases (lower to higher lot number), the distribution among replicate determinations becomes somewhat more symmetrical. This is reflected in the decreasing coefficient of variation (CV) with increasing mean level of analyte. Research has demonstrated a similar distribution of aflatoxin in contaminated corn, Brazil nuts (field contamination), and peanuts (post-harvest contamination).

As with all analytical procedures, the final result is obtained from a series of sequential steps. The uncertainty associated with the estimates that are generated (final results) is cumulative and contains contributions from each of the steps. Variance can be used as a parameter of this uncertainty. The total variance (V_T) for the overall testing procedure is equal to the sum of the variances from several sources:

$$V_T = V_S + V_{SS} + V_A \qquad [1]$$

where:

V_S = variance associated with the sampling procedure

V_{SS} = variance associated with the subsampling process

V_A = variance associated with the analytical method

Since variance is equal to the square of the standard deviation, it represents a measure of the precision (repeatability) associated with a process or a step; the most efficient way to make estimates of the levels of toxins more precise is to improve the repeatability of

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table

Results of Aflatoxin Analysis for 20 Replicate 2.27-kg Samples from Each of 10 Contaminated Lots of Cottonseed

Lot Number	Aflatoxin Test Results (µg/kg)																				Mean (µg/kg)	Variance	CV[1] (%)
1	0	0	0	0	0	0	0	0	0	1	1	1	1	1	3	5	7	9	10	14	2.7	17.1	156.0
2	0	0	0	1	1	1	1	1	1	1	4	6	10	10	12	13	16	27	40	44	9.5	174	139.6
3	0	0	0	0	1	1	1	1	1	1	8	9	12	23	24	24	25	30	40	50	12.6	234	122.0
4	0	1	1	1	1	1	1	4	4	11	12	14	19	20	21	22	24	28	38	56	14.0	223	107.0
5	0	4	9	14	16	16	25	27	30	30	31	32	32	32	34	37	40	42	42	100	30.3	819	94.5
6	6	6	7	14	20	22	24	24	31	33	38	40	42	45	54	60	67	68	68	165	41.7	1260	85.1
7	10	10	14	20	25	31	32	34	37	37	55	61	61	65	70	74	83	86	101	117	51.1	959	60.5
8	15	16	20	21	27	30	48	52	57	67	70	80	80	90	111	118	133	136	144	160	73.8	2183	63.4
9	1	16	29	40	53	73	85	89	100	104	113	118	120	121	121	143	157	175	260	266	109.9	4990	64.3
10	70	80	91	110	114	116	127	130	133	150	178	178	192	196	200	206	237	252	269	281	169.8	4741	40.6

From (52).
[1]CV = coefficient of variation = 100(Variance)$^{1/2}$/mean.

the step with the greatest variance. Improving the repeatability of steps that are not major contributors to V_T will not impact greatly on the magnitude of V_T. Studies of aflatoxin levels on granular (peanuts and cottonseed) produce have indicated that the sampling step, especially for smaller sample sizes, is the major source of error (uncertainty) in the overall analytical process. Sampling error is especially large because the aflatoxin is present on only a very small percentage of the kernels within the lot (<<0.1%), but the concentration within that kernel can be extremely high (up to 1 × 10^6 µg/kg). The one way to reduce V_S is to take a larger sample size. Table 20-4 presents estimates of the range of aflatoxin test results (at the 95% level of confidence) that can be expected for different composite sample sizes taken from a cottonseed shipment that is contaminated with 100 µg/kg of toxin. The predicted range of results does not decrease at a constant rate with increasing sample size, indicating that increasing the sample size beyond a certain point may not be the best use of resources. It is assumed that the sample is taken in such a way that all parts of the shipment have an equal chance of being included in the sample. The contaminated particles occur in isolated pockets that are unevenly distributed; a composite sample must be accumulated from many different locations throughout the shipment. One of the characteristics of sampling statistics is that the reliability of a random sample does not depend so much on the size of the shipment as on the size of the sample. Thus, as a first approximation, the size of the shipment can be ignored.

Once a composite sample has been obtained, the aflatoxins must be recovered, usually by a process of extraction. Not surprisingly, it is not feasible to extract the mycotoxins from the total sample, which is much too large to be handled conveniently. In practice a subsample is prepared. As with the sampling step, the reproducibility of the subsampling also is dependent on the mycotoxin concentration. One way to reduce V_{SS} is to take a larger subsample. However, there is a practical limit to the size of subsample that can be handled. If the commodity is granular, it is essential that the sample be comminuted (ground to a smaller particle size and mixed thoroughly) in a suitable mill. This comminution process not only reduces the variance by the particle size (more particles per unit mass), but it also increases the homogeneity of the product. The final particle size to which the sample can be reduced is limited by the screen mesh of the mill.

The variance associated with a particular analytical method for aflatoxins also is concentration dependent. The variance V_A can be reduced only by performing more replicate analyses (V_A is inversely proportional to the number of replicate analyses). Detailed sampling plans for separate commodities have been developed by AOAC International and AOCS and by FDA in collaboration with USDA.

For raw shelled peanuts, for which marketability is certified if the lot is found to contain less than the 15 µg/kg (sum of aflatoxins B_1, B_2, G_1, G_2) action level, a sequential sampling plan is recommended. It is considered that the 15 µg/kg action level will permit processors to meet the FDA's 20 µg/kg guideline for legal limits in finished products. In sequential sampling, a bulk sample of approximately 70 kg is accumulated (at a rate of one incremental portion per 225 kg of lot weight). This bulk sample is divided, in a random manner, into three 48-lb (21.8-kg) samples using a Dickens mechanical rotating divider. The first sample is ground in a special subsampling hammer mill fitted with a 3 mm diameter screen. A subsample (1100 g) is slurried in water and analyzed by thin-layer chromatography in duplicate. If the average of the two determinations is less than or equal to 8 µg/kg the shipment is passed and no further testing is performed. If the average is ≥45 µg/kg, the shipment is rejected. For averages between these two extremes, a second 48-lb sample is analyzed in duplicate and the average of the four results is used to decide whether to accept (≤12 µg/kg) or reject (≥23 µg/kg). If the average is between this second set of extremes, the third 48-lb sample also is analyzed and the average of the six determinations is used to decide whether the level of contamination is more or less than the action level of 15 µg/kg.

Typically, a lot of raw shelled peanuts is analyzed prior to shipment to a manufacturer. Alternative sampling plans are employed typically at the point of entry, by major importing countries. Guidelines of 10 µg/kg (total aflatoxin) and 4 µg/kg on raw and finished

Predicted Range of Test Results (at the 95% Level of Confidence) When Testing a Cottonseed Lot, That Is Contaminated with 100 µg/kg Aflatoxin

Sample Size (kg)	Low[1] Value (µg/kg)	High[2] Value (µg/kg)	Standard Deviation[3]	Range (High–Low)
1	0	271	87	271
2	0	222	62	222
4	13	187	45	174
8	37	163	32	126
16	53	147	24	94
32	65	136	19	72

From (52)

[1] Low = CC − 1.96 (std deviation). If low was <0 a 0 was recorded.
[2] High = CC + 1.96 (std deviation).
[3] Standard deviation is based on a single 100-g subsample taken from the sample and one analysis.

peanut products in the United Kingdom and 5 µg/kg represent the legal limit in The Netherlands, respectively. Actionable levels (which serve as a basis for the accept/reject decision in these two importing countries are 10 µg/kg (total) and 3 µg B_1/kg, respectively.

Aflatoxin contamination of foods of animal origin is not considered likely because livestock and poultry have the ability to dilute and detoxify these chemicals. The feed-to-tissue ratio for aflatoxin B_1 has been estimated to be 14,000 for beef cattle liver and 2200 for chicken eggs. By contrast, this ratio drops to between 100 and 200 for milk (from dairy cattle) for which the predominant aflatoxin is M_1 (a ring hydroxylated metabolite of B_1). FDA has established an actionable level of 0.5 µg/kg for fluid milk.

20.2.3 Chemical Screening Procedures

Nonquantitative procedures include minicolumn screening tests and single-dimensional TLC procedures that are intended to provide a preliminary indication of the presence of analyte mycotoxins. Since the majority of samples are anticipated to contain no detectable residues, a rapid yet sensitive screening procedure can identify those samples that will require a more time-consuming quantitative analysis. Minicolumn procedures exploit the native fluorescence of aflatoxins, zearalenone, or ochratoxin A analytes. Glass chromatography tubes (4–6 mm internal diameter) containing different adsorbants are available commercially in a prepacked format (Table 20-5) and are used in implied procedures to detect either the aflatoxins or zearalenone and ochratoxin A. After partial cleanup, the extract is added to the head of the column and then eluted with various solvents. In the final step, the analyte(s) are eluted from upper absorbing layers into a Florisil lover layer. When viewed under long wavelength UV radiation (365 nm), the analyte(s) appear as a blue fluorescent band. The method limit of detection is 10–15 ng/g. The precolumn sample treatment is somewhat matrix dependent (40).

In the past, screening corn for possible aflatoxin and other mycotoxin contamination using a "black light" was popular. However, the bright green-yellow fluorescence (under illumination with long wavelength ultraviolet light) is indicative of the presence of certain strains of Aspergillus, but does not indicate the presence or absence of mycotoxins. The fluorescence actually is produced by kojic acid, an innocuous metabolite of A. flavus.

TLC has been used frequently both for screening assays and separately for quantitation of mycotoxins. The advantages of using a TLC-based preliminary screen of samples include the fact that more samples can be assayed on the same plate because fewer stan-

dards are required. Quantitative assessments require a series of standard concentrations for each analyte. The screening procedure also can be used to provide a crude estimate of the levels of toxin so that the volume of extract can be adjusted to obtain a response that is within the linear range of a quantitative method subsequently applied to the same sample. The principle limitation of unidirectional TLC is the presence of coextractives that may interfere with the detection and quantitation. This limitation often is overcome by resorting to two-dimensional TLC; however, the number of samples or standards that can be run on the same plate is severely limited. There are numerous environmental factors that influence the relative mobility (R_f) of an analyte on a TLC plate, including temperature, the activity of the stationary phase, and the degree of solvent undersaturation of the chromatography tank. In consequence, many of the official methods do not report R_f values from their procedures. Authentic standards must be run frequently (if not with every sample) to ensure that the chromatographic conditions remains unchanged. Despite the increased variability associated with TLC relative to other analytical techniques, it is important to remember that, for mycotoxin analyses, the sampling error is probably the predominant contributor to the overall uncertainty associated with the final results. Multitoxin screening TLC procedures have been reviewed by Steyn et al. (41) and by Romer (42).

20.2.4 Quantitative Chemical Procedures

Quantitative chemical methods for mycotoxins are inevitably multistage methods that follow the same general procedures as for pesticide residues. These procedures typically involve separate steps of extraction, filtration, cleanup, concentration, chromatographic separation, detection/quantitation, and confirmation. Extraction procedures tend to be similar to those for pesticide residues. Aqueous methanol, acetone, and/or acetonitrile are added to the subsample and either blended at high speed or vigorously shaken for 0.5 hr. Defatting of lipid-rich food matrices is frequently required; typically extractions with hexane or isooctane can be performed prior to, during, or after the mycotoxin solubilization step. The use of dimethyl ether for defatting operations is avoided because of analyte (aflatoxin) losses.

After filtration to remove suspended solids, cleanup procedures are used to further purify the extract. In addition to preparative chromatographic columns or solvent partitioning, using procedures described in the pesticide residues section, an aqueous anionic precipitation procedure is used sometimes to remove plant pigments and proteinaceous substances.

The precipitation can be induced with a variety of additives including lead, zinc, and ammonium salts and phosphotungstic acid. This procedure is limited to those extracts in polar organic solvents containing more than 55% water.

After the partially purified extract is concentrated, it is subjected to chromatographic separation by two-dimensional TLC, HPLC, or, for certain classes of mycotoxins, by GC. An excellent overview of two-dimensional TLC procedures is provided by van Egmond (43). The most popular automated chromatographic technique for mycotoxins has been HPLC. Many of these toxins (trichothecenes are the exception) can be detected by ultraviolet or fluorescence spectroscopy with sufficient sensitivity to provide for quantitation at the low µg/kg levels. Reversed-phase chromatographic separation with pre-column treatment with trifluoroacetic acid (to convert aflatoxins B_1 and G_1 to their corresponding hemiacetals) or post-column derivatization with iodine has been used to increase the fluorescence response of these analytes (44) in aqueous mobile phases. For the trichothecenes, procedures often are optimized for the recovery of either the relatively less polar class A subgroup (diacetoxyscirpenol, T-2 toxin, HT-2 toxin, and neosolaniol) or the more polar B subgroup [deoxynivalenol (DON), nivalenol, and fusarenon-X]. The purified extracts are treated to convert the analytes to either their trimethylsilyl or their heptafluorobutyryl derivatives, and the derivatives are detected by electron capture GC (or less often by flame ionization) following separation on packed or capillary columns.

20.2.5 Biochemical Methods

20.2.5.1 Immunoassays

Although a variety of biological assays have been described that are useful in tracing sources of mycotoxin assays (acute toxicity to the larvae of brine shrimp or fish or to chick embryos), their use in the surveillance of foods and feeds is only of minor importance. By contrast, the potential for the determination of mycotoxins by immunoassay techniques has been amply demonstrated by the development of both radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) procedures for several mycotoxins. These include a number of aflatoxins (B_1, G_1, Q_1, and M_1), trichothecenes (DON, DON triacetate; T-2 toxin, diacetoxyscirpenol), ochratoxin A, zearalenone, and rubratoxin in a variety of food matrices. Limits of detection are in the picogram to nanogram range. The speed (10 min for the competitive binding step), simplicity (directly applicable to liquid samples and to water-methanol extracts of solid samples), and the reliability have been improved steadily. Estimates have suggested

that, excluding research and development, ELISA procedures cost only 6% and 3% as much as GC and GC-MS based procedures, respectively.

Kits for the detection and quantitation of aflatoxin and other mycotoxins (Table 20-5) have become available from several commercial sources. As suggested by Pestka (44), answers to a number of questions should be considered in choosing a source of an assay kit:

1. Are the limits of detection and dynamic range of the assay relevant to the needs of the laboratory? If the anticipated levels of mycotoxin residues are high, extensive dilution of the extract might be necessary for quantitation. Since the absorbance recorded in an ELISA procedure is inversely related to the logarithm of analyte concentration, measurements made near the middle of the standard S-shaped curve will be more precise than measurements at either extreme. It would be advantageous to have the concentration corresponding to the actionable level at the midpoint of the calibration curve.

2. In view of the extreme heterogeneity of contamination, are realistic sampling protocols described in the literature accompanying the product?

3. Since the antibodies also might react with closely related chemical structures, what is the cross-reactivity of each analog relative to the target analyte? Antibodies developed against aflatoxin B_1 will cross-react with aflatoxin B_2. [However, the strength of the binding (avidity) will be different for the two aflatoxins.] Typically, analog competition curves do not overlap and might not have similar slopes. Moreover, the specificity of the antibodies might vary from batch to batch. Ideally, antibody lot characteristics should be defined in terms of limit of detection and sensitivity range for the analytical procedure, resistance to organic solvents, and the variability of the antibody-coated solid support.

4. Since certain kits come in a modular format with assay units that can be physically separated, can the kit be used to analyze a single sample? By contrast, other kits are designed for large numbers of samples that are analyzed simultaneously.

As an example, an enzyme-linked immunosorbent screening method for aflatoxin B_1 in cottonseed and mixed feed that is applicable to screening B_1 at ≥ 15 µg/kg has been adopted first action as a joint AOAC-IUPAC method. Antibodies (to aflatoxin, coated onto plastic microliter wells, lyophilized horseradish peroxidase-conjugated aflatoxin B_1, an enzyme

20-5
table   Some Commercially Available Mycotoxin Test Kits and Their Sources[1]

Neogen Corporation 620 Lesher Place Lansing, MI 48912 (http://www.neogen.com)	Test kits are available to screen (Agri-Screen™ kits) or quantify (Veratox™ kits): aflatoxins, zearalenone, ochratoxin, fumonisin, vomitoxin (DON), T-2 toxin or ochratoxin. Antibodies specific for the analyte mycotoxin are chemically bound to the wall of a microwell. Added mycotoxin–enzyme conjugate competes with micotoxin in the extract for antibody binding sites. After incubation, unbound materials are washed from the well. Finally, bound enzyme mediates a color forming reaction with an added substrate.
Romer Labs Inc. 1301 Stylmaster Drive Union, Missouri 63084 (http://www.romerlabs.com)	AflaCup™ test kits use solid-phase immunoassay techniques for B_1, B_2, and G_1. Mycotest™ kit provides a simultaneous assay for B_1, zearalenone, and deoxynivalenol or for DON and two acetylated transformation products. MycoSep™ columns are used to purify crop extracts prior to derivatization coupled with HPLC, GC-MS, fluorimetry, or TLC.
Vicam, LP 312 Pleasant Street Watertown, MA 02172 (http://www2viacam.com/VI CAM/)	Test kits are available for: Aflatoxins (B_1, B_2, G_1, and G_2 in feeds, foods, grains, and nuts and M_1 in dairy products) Zearalenone, ochratoxin, fumonisin An immunoaffinity column technology is used to bind analytes onto the antibody beads. Analytes are recovered in methanol, derivatized and quantified by fluorimetry.
Editek Inc. P.O. Box 908 1238 Anthony Road Burlington, NC 27215 (http://www.editek.com)	Test kits (EZ-Screen™ Quik-Card™) are available for the detection or quantitation (EZ-QUANT™) of aflatoxins ochratoxin, T-2 toxin, or zearalenone. A competitive enzyme immunoassay is performed on a solid support (Quik-CARD) impregnated with rabbit antibodies to the analyte. Horseradish peroxidase bound to the analyte competes with analyte from the sample for antibody binding sites. After removing unbound materials an added colorless substrate is colored by reaction with the bound peroxidase. An analogous competitive binding assay forms the basis of the EZ-QUANT™ system.

[1]Note: Absolute detection limits of these test kits will vary and should be examined in relation to the needs of the user.

substrate [2,2'-azino-d-(3-ethyl-benzthiazoline sulfonate)] (ABTS) in pH 4 citrate buffer, hydrogen peroxide (30%) in pH 4 citrate buffer, and a color stopping solution are available as an Agri-Screen for Aflatoxin kit (Neogen Corp.). Similar kits are available from Romer Labs, Vicam LP, and from Editek Inc. Several companies in Great Britain also have developed and distribute kits.

20.2.5.2 Immunoaffinity Separations

A further very promising development is the use of antibodies to isolate aflatoxins from a biological matrix. This potentially simple approach can result in a degree of cleanup that is superior to the more elaborate standard methods. Commercial single-use columns consisting of anti-aflatoxin antibodies covalently bound to a gel matrix (a beaded agarose) in a plastic cartridge have been introduced. The column packing material selectively adsorbs aflatoxins from coextractives in a crude extract serving as a concentration technique. The analytes then are readily released from the packing with a polar organic solvent.

One application involves the use of monoclonal antibody affinity chromatography as a one-step column cleanup prior to the fluorometric determination of aflatoxin M_1 in milk (45). Raw milk samples (40 ml) were mixed with NaCl (1 g), centrifuged at 2000 x g for 5 min, and then filtered. A 25-ml aliquot of the filtrate was passed through a column (Aflatest™, Vicam LP) under a slight positive pressure. The column was washed with two successive 10-ml portions of 10% (vol/vol) methanol; then the analyte was eluted with 1 ml of 80% methanol. The eluate was diluted with 1 ml

of aqueous bromine, and the fluoresence measured at 450 nm (λ_{ex} = 360 nm). Recoveries at the 0.05–2 µg/liter levels were excellent. A method detection limit was estimated to be 0.05 µg/l (tenfold less than the FDA actionable level).

One appreciable advantage of this general approach is that it circumvents the rather narrow linear dynamic range of analyte concentrations associated with ELISA and RIA methods. A concise overview of the techniques for preparing immunoaffinity columns has been published (46).

20.3 DRUG RESIDUES

20.3.1 Introduction

In addition to the drugs administered at therapeutic levels to combat diseases in food producing animals, the subtherapeutic use of antimicrobial drugs also has played an important role in animal husbandry. FDA approved the addition of subtherapeutic levels of antibiotics to animal feeds almost 40 years ago. These levels (1) reduce the incidences of infectious diseases caused by bacteria and protozoa (prophylactic effect), (2) increase the rate of weight gain of treated animals, and (3) decrease the amount of feed needed to achieve these weight gains. The continued use of subtherapeutic levels of antibiotics can pose a serious but indirect hazard to humans. Abuse of this practice has resulted in the dominance of antimicrobial-resistant enteric bacteria in some food animals and can result in the transmission to the human reservoir of bacterial resistance to antimicrobial agents.

The use of antimicrobial agents in feeds is strictly regulated—monitoring of compliance is the responsibility of the Center for Veterinary Medicine (CVM) of FDA. A tolerance in edible tissues has been set for each antibiotic and sulfonamide approved for use in animal feeds. By analogy to pesticide registration, a tolerance, based on uncooked edible tissues, is established after an extensive review of the toxicology, chemistry, and biochemistry of the active product and the development (by the sponsors of the application) of an analytical method for determining residues of the drug in tissues. As part of the approval process, restrictions on the dosage level and duration, species that may be treated, and the withdrawal period (the time between the last availability of the drug to the animal and slaughter or the use of milk or eggs by humans) are established. The withdrawal period can vary between 0 and 30 days. Tolerances, if established, represent total residues (parent compound plus all compounds derived from it, including metabolites, conjugates, and residues bound to macromolecules). In an effort to reduce the number of methods required for monitoring, the concepts of a marker residue and a target tis-

sue have been advanced. The marker residue is a selected residue (possibly the parent compound) that has a known relationship with the level of total residues in each of the edible tissues. The traditional method of establishing this relationship is by performing a feeding trial (under conditions of the proposed use of the candidate drug) with a radiolabeled parent compound and monitoring the depletion and fate of the label with time. Based on the pharmacokinetics of the drug and its subsequent depletion from the different tissues with time, an estimate of the levels in tissues at sacrifice can be obtained.

Estimates are that, at some time during their lives, nearly 80% of poultry, 75% of swine, 60% of feedlot cattle, and 75% of dairy calves have been fed with antibiotics (47). The Food Safety and Inspection Service (FSIS) of USDA monitors edible tissues destined for commerce for residues of drugs, pesticides, industrial chemicals, and heavy metals. The United States Code of Federal Regulations lists tolerances for some 80 animal drugs in foods, of which some 30 (mostly antibiotics) are readily detected by microbiological screening assays. By contrast to pesticide multiresidue procedures, chemically based multiresidue procedures for drug residues tend to be more limited in scope in that they are directed at specific classes of drug residues.

20.3.2 Screening Assays for Antibiotic Residues

Traditional screening assays have relied on the inhibition of growth of microorganisms by antibiotic residues present in the test sample. These assay procedures are based on either diffusion processes or on turbidity. The growth of an indicator organism, in a transparent liquid culture, can be followed by monitoring the increased turbidity with time. In diffusion-dependent assays, the material to be assayed diffuses through an agar-based nutrient medium that has been uniformly seeded with spores of a susceptible organism. Upon incubation, a zone of inhibition of germination and growth develops, indicating the presence of inhibitor(s). There are several factors that affect the size and appearance of zones of inhibition. The number of viable organisms used to inoculate the medium is critical because the density of growth (and therefore the visualization of zones) is dependent on the initial numbers of organisms. The temperature of incubation also must be rigidly controlled because both the rate of growth of the organism and the rate(s) of diffusion of inhibitor(s) are temperature-dependent phenomena. Porosity of the medium also influences the rate of diffusion. In general, lower proportions of agar result in larger zones of inhibition. Other factors include the depth of the agar layer, the age of the inoculum, the technique of adding the sample to the plate, and

the presence of coextractives from the sample. Both turbidity and diffusion-based techniques can be carried out manually, or many of the steps can be automated. A detailed monograph on the theory and application of microbial assays has been prepared by Hewitt and Vincent (48).

Two procedures, STOP (swab test on premises) and CAST (calf antibiotic and sulfa test), are typical of microbiol assays for antibiotic residues. Cotton swabs that have been used to sample the suspect tissue are placed in contact with gelled growth media that has been amended with *Bacillus subtilis* (ATCC 6633) spores and incubated at 32°C for 16–18 hr (STOP) or *Bacillus magterium* (ATCC 9885) and incubated at 44°C (CAST). If positive, these screen are usually followed by a thin-layer bioautography assay.

There are some 40 drugs approved for use in lactating dairy cows, 11 of which have been approved for the treatment of mastitis and respiratory infections. Raw milk is screened routinely for antibiotic residues; antibiotic-contaminated milk (and milk products) are considered as adulterated by FDA. The residues of greatest concern include penicillin, ampicillin, cephapirin, hetacillin, and amoxicillen, all of which can cause hypersensitivity reactions for certain consumers. Antibiotic residues can interfere with the acid and flavor production during the manufacture of buttermilk and similar products, the acid production of starter cultures used in processed milk products and cheeses, and starter culture growth when propagated in reconstituted skim milk.

In total, screening assays for antibiotic residues in milk are efficient monitoring procedures in that they are simple and rapid (permitting numerous samples to be screened). However, they are nonspecific and respond only to biologically active residues that inhibit the growth of the indicator organism. Prior to 1991, the one official test method for drug residues in raw milk was the *Bacillus stearothermophilis* disk assay (BSDA). Since then, continued improvements to and testing/approval of kits have resulted in a wide choice of commercial screening kits (Table 20-6). However, there is no ideal kit currently available for detecting antimicrobial drug residues in raw milk.

Screening tests do not identify the specific analyte that causes the positive response nor do they measure the quantity of residue(s). A positive result from a screening test is a presumptive indication that one or more analyte is present. None of the β-lactam tests can detect all six approved β-lactam drugs. Screening tests have limits of detection for specific drug residues that are below the tolerance level. Thus, screening assays can provide false violation results (i.e., they can correctly provide positive results for levels below the tolerance level permitted by legislation). In addition, each kit fails to detect residues of one or more approved β-

lactams at their tolerance level. Finally, natural defense secretions in milk from mastitic cows have produced a positive response in antimicrobial screening assays even though the cows had not been treated with any animal drug. However, the positive responses from these natural inhibitors occur only under conditions in which the somatic cell count is many times greater than the maximum number of somatic cells permitted under current legislation. In addition, it seems unlikely that whole herds would be affected sufficiently to produce a positive response to tanker truck loads of raw milk.

The Charm II test (Charm Science) is a rapid radioisotopic assay procedure that can detect the following antibiotic groups: β-lactams, tetracyclines, macrolides, aminoglycosides, novobiocin, sulfonamides, and chloramphenicols. The assay is based on a competition between labeled drug and residues in the milk sample for a limited number of specific binding sites on the surfaces of bacteria that are added to the sample. In brief, the procedure involves the addition of the radiolabeled tracer antibiotic and the binding microorganism to the milk sample, a short incubation, centrifugation, fat removal, resuspension of the microbial plug in a scintillation fluid, and counting. The greater the concentration of antibiotic residue, the less radiolabeled tracer will become bound to the microorganism. Two antibiotic groups can be assayed in each tube by using a combination of ^{14}C and ^3H tracer antibiotics. The various antibiotic types can be assayed in approximately 12 min, and with limits of detection that are very much lower than the more traditional diffusion assays. For β-lactam antibiotic residues, Angenics Inc. has developed a rapid (6-min) antibody-based assay, in kit form, that is performed on a glass slide and evaluated in a monitoring device. Penzyme On-Farm and Laboratory III tests (Smithkline Animal Health Products) are enzyme-based colorimetric assays for β-lactam antibiotics.

20.3.3 Chemically Based Approaches To Quantitative Determinations

20.3.3.1 Overview

The approaches to the quantitative determination of drug residues in tissues, in feeds, or in other food products follow the same general steps as for other trace analytes. After an optional sample pretreatment (to aid in the release of bound residues), drug residues are solubilized with an appropriate solvent, the crude extract is purified by partitioning or column chromatography, and then the purified extract is analyzed using an automated chromatographic technique. However, for drug residues, cleanup procedures often involve acid–base partitioning against organic solvents to take advantage

20-6

| table | Examples of Commercially Distributed Screening Kits for Drug Residues |

Agridiagnostics/Editek Inc., 1238 Anthony Road, Burlington, NC	*Swab Test on Premises* (S.T.O.P.) detects β-lactams, tetracy-clines, aminoglycosides, and macrolytes. If the tissue contains antibiotic residues, fluid from the tissue swab will inhibit the growth of a sensitive organism on a culture plate. *Calf Antibiotic Sulfa Test* (C.A.S.T.) detects β-lactams, tetracy-clines, aminoglycosides, macrolytes, and sulfa drugs. Analytes will diffuse out from the swab and inhibit the growth of bacterial colonies in the area around the head of the swab. *Fast Antibiotic Swab Test* (F.A.S.T.) detects β-lactams, tetracy-clines, aminoglycosides, macrolytes, and sulfa drugs. Identical to the C.A.S.T. assay except that a color change is generated in approximately 8 hr. *Live Animal Swab Test* (L.A.S.T) detects β-lactams, tetracy-clines, aminoglycosides, and macrolytes using a modification of the S.T.O.P. test to permit the monitoring of urine. *Sulfa on-Site* (S.O.S.) employs a TLC technique.
Charm Science 36 Franklyn Street Malden, MA 02148-4120 *http://www.world.std.com/~charm1/chrmweb.htm*	*Charm 1, Farm Test, RITe Test, B.st Tablet Disk Assay.* Screening kits for β-lactam, sulfonamides, tetracycline macrolide, and aminoglycoside drug residues in milk, beef, pork, eggs, fish, feed, and grain honey serum and urine (not all kits are applicable to all matrices). *Charm II* detects novobiocin, spectinomycin, and folic acid analog residues in milk, chloramphinicol residues in the matrices above, and performs all other Charm tests.
IDEXX Laboratories Inc., 1 IDEXX Drive Westbrook, ME 04092 *http://www.idexx.com*	*SNAP β-lactam, SNAP Tetracycline.* A competitive binding assay coupled with enzyme-mediated color development within a card and card holder. The card also contains a positive control. *CITE Sulfa Trio, Probe Gentamicin*

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of the acidic character (phenolic compounds) or the basic character (benzimidazoles, sulfonamides, tetracyclines) of the analytes. For ionic analytes, ion-exchange cleanup columns have been used extensively. Alternately, the acid/base character of analytes can be exploited by performing the initial extraction with mineral acids (HCl, HClO₄) or with aqueous buffers to recover tetracyclines from meats, fish, and blood. Buffers sometimes can be combined advantageously with a water-miscible organic solvent to improve the selectivity of the solubilization step. The addition of the water-miscible organic solvent appreciably reduces the solubility of proteinaceous materials and avoids a separate deproteination step. Other advantages of this approach are that the procedure is simple and rapid, appears to be widely applicable, and results in consistently high recoveries. However, the resulting extracts cannot be injected directly onto a reversed-phase HPLC column for lack of analyte retention. The organic solvent is usually removed (in part or totally). One approach that can be effective for polar analytes (tetracyclines) is to add a nonpolar water-immiscible solvent (CH₂Cl₂ or hexane). In contrast to most pesticides, the polar drug residues are retained in the aqueous phase.

Analytes in the crude aqueous phase can be concentrated on a solid-phase extraction (SPE) cartridge or directly on the head of an HPLC column.

20.3.3.2 Automated Chromatographic Separations

As is the case for mycotoxins, the most popular automated approach to separation/quantitation of drug residues has been HPLC. The major applications of this approach have been for confirmatory rather than for screening purposes. One of the advantages of HPLC relative to GC is that frequently little sample preparation is required. A multiresidue procedure for eight benzimidazole residues in liver and muscle is typical of more recent developments in drug residue methodology (49). In brief, previously blended and frozen tissue sample (bovine, ovine, or swine liver or muscle), 10 g; Na₂SO₄, 5 g; 4 M K₂CO₃, 1 ml; and ethyl acetate, 30 ml are blended, and the filtrate is evaporated. The residue, in hexane, is partitioned against ethanol–0.2 M HCl. An aliquot of the aqueous phase is alkalized with K₂CO₃, and then purified on a C₂ minicolumn. The analytes are recovered from the minicolumn with ethyl acetate. The

organic solvent is evaporated, and the residues are resuspended in ethanol–ammonium phosphate mobile phase, separated by reversed-phase HPLC, and detected at λ_{298} nm. The identity of residues can be corroborated by GC–MS after hydrolyzing a second aliquot of the extract with HCl and derivatizing the liberated amine(s) with N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide. Recoveries, at the 100 µg/kg spiking level, averaged 88% ± 6%.

An HPLC screen for 10 sulfonamides in raw bovine milk is also illustrative (50). Sample preparation is minimal and involves partitioning the residues into chloroform–acetone, evaporating the solvent, resuspending the residues in 0.1 M potassium dihydrogen phosphate, and subsequently removing of fatlike coextractives with hexane. After filtration, the aqueous phase is analyzed by reversed-phase HPLC (using either of two isocratic mobile phases) with detection at λ_{265}nm. The coefficients of variation were 3–13% at the 10 µg/kg level of spiking. A recent overview of the application of HPLC to antibiotic residue determinations has been provided by Moats (51).

20.4 SUMMARY

As a society, we expend enormous effort and considerable resources to identify and control "synthetic" chemicals that are considered to pose a carcinogenic risk to society of greater than one in a million. The detection and determination of traces of pesticide, mycotoxin, and antibiotic residues in foods represents a formidable challenge for the analyst. In support of existing legislation, detailed sampling protocols and analytical procedures have been developed both to screen for and to measure toxic residues at trace and ultratrace levels. Despite a generation of dedicated research and many thousands of analyses per year, the level of consumer confidence in our food supplies appears to be declining somewhat. In response to concerns regarding the safety of our food supplies, newer methodologies will have to be developed and optimized to screen even greater numbers of samples and analyze for greater numbers of toxicants. Above all, we have to continue to ensure the rapid publication in the open literature of the results of monitoring programs to help allay consumers' concerns.

20.5 STUDY QUESTIONS

1. What is meant by the "tolerance level" for a pesticide on a fresh agricultural product?
2. Why is it that all analytical methods provide only estimates of the level(s) of an analyte within the sample? In your opinion, how exact are these estimates?
3. Briefly outline the five major steps involved in a quanti-

tative single-residue method for residues of a pesticide in a fresh plant food.

4. What strategies can be followed in an attempt to corroborate the presence of a pesticide, a mycotoxin, or a drug residue in a sample? What is the value, if any, of these approaches?
5. Conventional pomiculture can require up to 12 spray treatments of an orchard during a single growing season. The use of a "stop drop" agent can cause much of the crop to ripen at the same time to facilitate mechanized harvesting. You have been asked to ensure that the application of a registered stop drop agent "X" conforms to all existing regulations. How would you proceed?
   a. Which government agency is responsible for registering agent X on apples and which agency is responsible for ensuring that residues at harvest conform to existing tolerances?
   b. You find this pesticide and its tolerance level on apples in the "Compendium of Registered Pesticides." What is meant by "tolerance level?"
   c. In Volumes I and II of the Pesticide Analytical Manual you find multiresidue methods, single-residue methods, and screening methods. Which one of these types of methods would you use to ensure compliance with the tolerance level for agent X and why would you choose this approach over the other two classes of methods?
6. For immunoassay-based analytical methods, what is meant by the term crossreactivity?
7. Why haven't microbiological assays been developed to detect the presence of toxicogenic fungi in fresh or stored produce and used as an indicator of possible mycotoxin contamination of that product?
8. Why are sampling procedures for pesticide residues appreciably different from sampling procedures for mycotoxins even when dealing with the same sample matrix?
9. What are the advantages and disadvantages of analytical screening procedures for pesticide, for mycotoxin, and for drug residues?
10. Selected food products can be screened for certain mycotoxin residues using a simple minicolumn and for antibiotic residues by using a commercial kit such as the Charm II test. A different approach for aflatoxin or for certain sulfonamide antibiotic residues would be to perform an HPLC separation coupled with a selective, yet sensitive, detection process. Briefly explain how each of these three methods might be applied to solve these analytical problems. Include in your answer a description of the principles involved and the limitations of each approach. (See also Chapters 31 and 32 for general column chromatography and for HPLC, and Chapter 21 on immunoassays.)
11. Having suffered through this chapter, are you any closer to deciding whether foods are safe?

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Immunoassays

Deborah E. Dixon

21.1 INTRODUCTION

Immunological methods are finding widespread application in food analysis. The classical methods used in food analysis consisted of agglutination and gel precipitation reactions and gave way to the isotopic assays, such as radioimmunoassay (RIA). This assay provided excellent sensitivity, but the need for expensive equipment and radioisotopes presented a drawback to its widespread use. Evolution of enzyme immunoassays (EIAs) overcame the undesirable features (e.g., potential health hazards from exposure to radioactivity) posed by RIAs. Enzyme-linked immunosorbent assays (ELISAs) have found widespread use in the food industry. They can be used to detect desirable as well as undesirable substances. Questions have been raised recently about the safety of the food supply. A great deal of attention has been placed on the detection of undesirable substances such as pesticides, drug residues, hormones, growth promoters, microbial toxins, either mycotoxins or enterotoxins, natural intoxicants such as alkaloids, and other undesirable additives. Recent regulations put in place by the United States Department of Agriculture (Hazard Analysis and Critical Control Point, HACCP) require testing of seafood, meat, and poultry for *Escherichia coli* and *Salmonella* in processing plants and slaughterhouses. These tests must be rapid, provide robust performance in field situations, and be sensitive and specific to detect the undesirable pathogens. Immunoassays will find widespread application in these settings. Immunoassays continue to gain presence in testing of food for potential allergens, such as the protein gluten (e.g., gliadins) in wheat used to make gluten-free products for coeliacs.

There are a large number of chemicals present in foods that are natural components (e.g., carbohydrates, proteins, fats, color, flavor, minerals), as well as additives added intentionally to enhance processing. Concentrations for one or more of these may need to be monitored on a regular basis (e.g., for nutritional labeling). Microbes may produce beneficial compounds for food processing (fermentation for preservation). They also may be the source of harmful mycotoxins.

Immunological methods and most definitely ELISAs provide sensitivity, specificity, speed, and cost effectiveness that many of the classical microbiological and chemical analytical methods lack. The microbiological and chemical methods are costly and time consuming, requiring extensive sample preparation (e.g., extraction, cleanup procedures, concentration and separation steps), trained personnel, and expensive equipment. Immunoassays lend themselves to routine analysis of large numbers of samples. They can be used in the field, where qualitative screening often is desired. They also can be used quantitatively to obtain a value of how much of the analyte is present in the sample. ELISAs can be used in the private sector as well as government lab-

oratories as research tools. They also can find application in routine surveillance and quality control testing in manual or automated systems.

Until the mid-1970s, immunoassays were developed with polyclonal antibodies. Milstein and Kohler (1) developed hybridomas that secrete monoclonal antibodies. Monoclonal antibodies now have been developed for use in food analysis.

Principles of immunoassays and their applications for research and commercial use are discussed in this chapter. There has been an explosion of immunoassay development within the last few years, for both research and commercial product development related to food testing and food safety. Consumer awareness has heightened and the public is demanding a safer food supply, which now can be addressed by using technologies such as immunoassays that are sensitive, rapid, and specific. Much of the information on immunoassays is accessible via the Internet. Reference is made in this chapter to web sites to obtain the most recent information available on development and application of immunoassays.

21.2 PRINCIPLES AND PROCEDURES

21.2.1 Immunological Definitions

21.2.1.1 Antibodies

Antibodies are members of the family of immunoglobulins. These proteins are slightly glycosylated and exhibit a number of important and diagnostic features (2,3). There are two types of antibodies: polyclonal antibodies and monoclonal antibodies. In brief, to produce polyclonal antibodies, a properly selected antigen is injected into the host animal. The animal's immune system will recognize the antigen as a foreign substance and respond to it. The resulting antibodies are a mixture. There are a number of different determinants or one repeating determinant. There are various determinants to which the immune cells respond and result in a mixture of antibodies to those determinants. If a successful preparation is made, the population will contain some antibodies whose affinity and avidity are great for the foreign protein.

Monoclonal antibodies are secreted by hybridomas that are created by fusing hyperimmunized spleen cells, usually from mice, to myeloma cells, usually of mouse origin. The secreted antibodies are homogenous, since all the cells in the culture that secrete the antibodies originated from one cell.

21.2.1.2 Antigen

The term antigen is more complex in meaning than is the term antibody. The antigen may be described as the substance to which the antibody binds. The work of

Landsteiner (4) demonstrated that the antibodies bind to and have specificity for fairly small chemical moieties. Kabat estimated the chemical group may be the size of a pentasaccharide (5), while another study estimated it to be as large as a tetrapeptide (6). Therefore, an antigen can be a large soluble protein, a mammalian cell, or an organism (e.g., bacteria or virus). The actual site of antibody binding is called a determinant (7) or an epitope (8).

21.2.1.3 Hapten

The term multivalent can be used to describe antigens made of proteins or bacteria. Small hormones and haptens are examples of univalent antigens. A hapten is a small molecule of less than 1000 daltons. It is nonimmunogenic in its own right and must be chemically linked to proteins (in vivo or in vitro) to produce antibodies. When a small molecule is attached to a large carrier molecule [e.g., bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH)], the immunogen is called a conjugate antigen.

21.2.2 Methodology for Immunoassays

Immunoassays (IAs) comprise a test format that is antibody based. They can be used to detect antigens or antibodies. They can be developed for detection of large or small molecules.

21.2.2.1 Isotopic Immunoassays

21.2.2.1.1 Overview In an isotopic immunoassay, hapten or antigen can be measured. The assay is based on competition for antibody between a radioactive indicator antigen and its unlabeled counterpart in the test sample. As the amount of unlabeled antigen in the test sample increases, less labeled antigen is bound. The concentration of antigen is the test sample can be determined from comparison with a standard calibration curve prepared with known concentrations of purified antigen (9).

21.2.2.1.2 Radioimmunoassay (RIA) The sensitivity of the RIA is largely limited by the amount of radioactivity that can be introduced into the radiolabeled antigen (9). Levels as low as 1 ng can be detected when carrier-free radioactive iodine-125 is used as an extrinsic label. Precipitates usually do not result due to the extremely low concentrations used. There are several procedures that can be used to separate free and bound indicator antigen. In the classical method, complexes of antibody bound to radiolabeled antigen are precipitated with antiserum prepared against the antibody moiety (anti-species antibody).

There also are some solid-phase assays that alleviate the need for the second anti-species antibody (Fig.

21-1). In one method, antibodies to the antigen are adsorbed onto the plastic (e.g., polystyrene) tube. Next, radiolabeled antigen binds specifically to the adsorbed antigens and can be counted. When unlabeled antigen that competes also is present, less radiolabeled antigen can be proportionally bound. The unbound fraction that is left can be removed. This method is cost effective, quick, and highly sensitive. For example, it is possible to detect less than 0.001 μg of antigen when a tube is coated with 1 μg of antibody (10).

21.2.2.2 Nonisotopic Immunoassays

Nonisotopic immunoassays are different from isotopic immunoassays mainly due to the type of label used, the means of endpoint detection, and the possibility of eliminating a separation step. Two types of nonisotopic immunoassays are fluoroimmunoassays (FIAs) and enzyme immunoassays (EIAs).

21.2.2.2.1 Fluoroimmunoassays Fluorescein and rhodamine are used commonly for labeling molecules. There are three types of fluoroimmunoassays: (1) nonseparation fluoroimmunoassays that require no separation step of bound from unbound product, (2) polarization fluoroimmunoassays that operate based on antibody binding to enhance the signal, and (3) quenching fluoroimmunoassays that depend on a decrease in signal from the bound fraction (Fig. 21-2). The quenching of the activity is attributed to the antibody's ability to affect the excitation or the emission from the labeled small molecule (12).

21.2.2.2.2 Enzyme Immunoassays (EIAs) EIAs employ enzyme labels and are divided into two categories: homogeneous and heterogeneous. Homogeneous assays require no separation of unreacted reagents because the immune reaction affects the enzyme activity. Heterogeneous assays have separation steps. ELISA, a type of heterogeneous assay, requires washing between each step to remove unbound reagents. Enzyme labels widely used include alkaline phosphatase, glucose oxidase, and horseradish peroxidase. These enzymes catalyze reactions that cause substrates to degrade and form a colored product that can be read either spectrophotometrically or visually by eye. Depending on format, either antibody or antigen is adsorbed onto a solid phase, which can be polystyrene tubes, polystyrene microtiter wells, or membranes (i.e., nitrocellulose and nylon) (13, 14).

1. Sandwich ELISA. This format enables detection of large antigens (bacterial, viral, and other large proteins). Two preparations of antibodies are used, one to coat the solid phase and one onto which the enzyme is attached (Fig. 21-3). These antibodies can have the same specificity or can be directed against separate
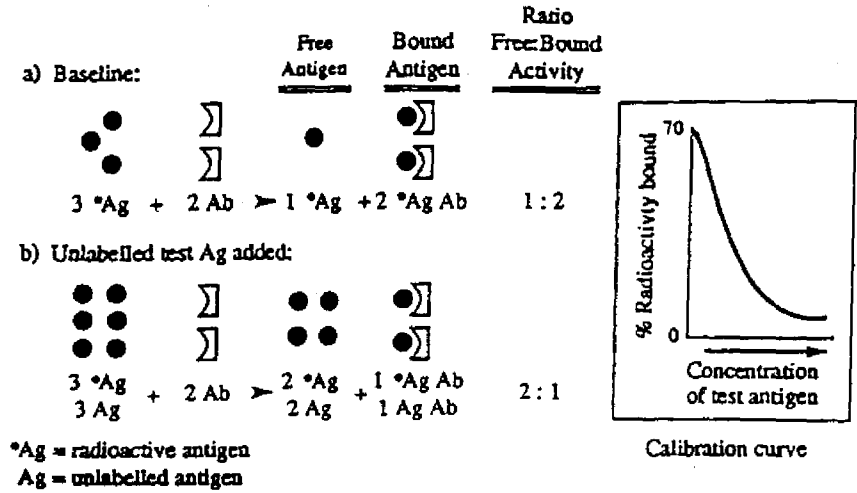
21-1 figure Radioimmunoassay methodology. Principle of radioimmunoassay (simplified by assuming a very highly avid antibody and one combining site per antibody molecule). (a) If we add 3 moles of radiolabeled Ag (•) to 2 moles of Ag, 1 mole of Ag will be free and 2 bound to Ab. The ratio of the counts of free to bound will be 1:2. (b) If we now add 3 moles of unlabelled Ag (•) plus 3 moles of Ag to the Ab, again only 2 moles of total Ag will be bound, but since the Ab cannot distinguish labeled from unlabeled Ag, half will be radioactive. The remaining antigen will be free and the ratio of free:bound radioactivity changes to 2:1. This ratio will vary with the amount of unlabeled Ag added, and this enables a calibration curve to be constructed. [From (11), used with permission.]
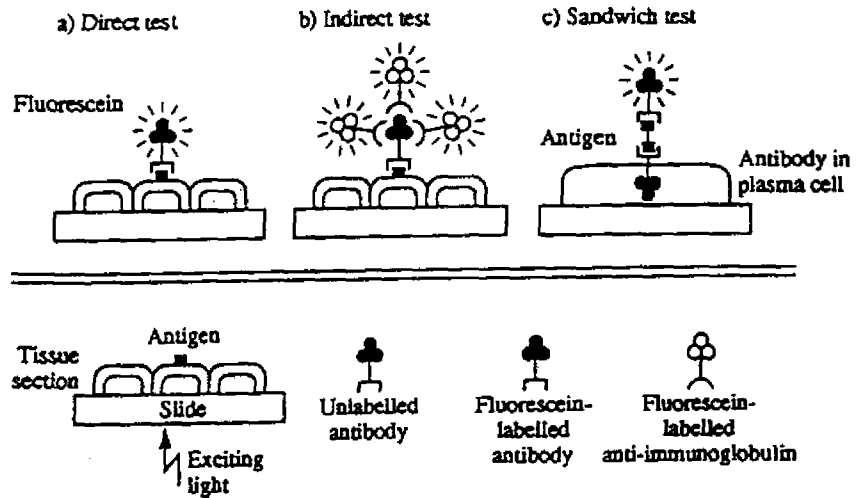
21-2 figure Immunofluorescence methodology. The basis of fluorescence antibody tests for identification of tissue antigens or their antibodies. • = fluorescein labeled. [From (11), used with permission.]

antigenic determinants. They can be raised in the same or different species. The color development is directly related to the amount of antigen present. The assay derives its name from the position the antigen occupies in the test. It is sandwiched between the unlabeled antibody attached to the solid phase and the enzyme-labeled antibody, which is added following addition of the antigen. A washing step is required between each step to remove unbound reactants.

An indirect double-sandwich ELISA may be developed. Here the solid-phase antibodies are specific

to the large protein produced in species A (e.g., rabbit) are coated into the solid phase. Serial dilutions of standards and sample(s) are added and the mixture is incubated for a specified time. Unbound antigen is washed out and a fixed amount of specific antibody from species B (e.g., mouse) is added. Following incubation of the second antibody and a washing step, a labeled antibody (anti-species B antibody) is incubated and then the excess washed out. Substrate is added and color development of the amount of antigen present also is proportional (14).

Competitive assay using enzyme-labelled antigen

Tubes with
adsorbed
antibody   Add labelled antigen   Add enzyme substrate

A) with food extract
B) without food extract

Color

Antigen conc.

Competitive assay using enzyme-labelled antibody

Adsorbed
purified
antigen   Add labelled antibody   Add enzyme substrate

A) with food extract
B) without food extract

Color

Antigen conc.
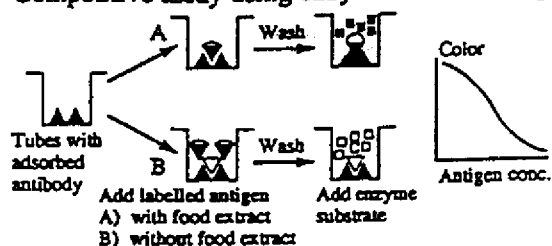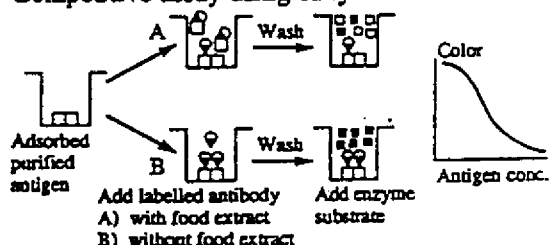
Double antibody sandwich assay

Adsorbed
antibody   Food extract and labelled antibody added   Add enzyme substrate

Color

Antigen conc.
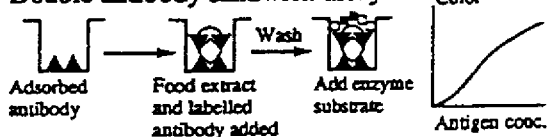
21-3
figure

Sandwich ELISA methodology. Enzyme-immunoassay formats used for quantification of food protein antigens. [From (15), used with permission. Illustration from *Development and Application of Immunoassay for Food Analysis*, J.H. Rittenburg, Ed., copyright © 1990 by Elsevier Science Publishers, Ltd., reprinted by permission of publisher.]

2. Competitive Direct ELISA. In this format, free hapten competes with an enzyme-labeled hapten for a number of limited antibody sites attached onto a solid phase. Unbound reactants are washed before substrate is added. Following substrate addition, the color is produced is indirectly related to the amount of hapten in the test sample (Fig. 21-4).

3. Competitive Indirect ELISA. In this format, antibody is free in solution, and competition occurs between free hapten and solid-phase antigen bound to the plastic or membrane. Following an incubation step, unreacted reagents are washed away, and an anti-species antibody labeled with enzyme is added and incubated. After another washing step, substrate is added. The color produced is indirectly related to the amount of hapten present in the sample (Fig. 21-5).

21.2.2.3 Other Immunochemical Methods

21.2.2.3.1 Agglutination Particle immunoassay can be defined as an assay based on immunological agglutination in which either an antigen or its corresponding

antibody is attached to an inert particle (Fig. 21-6). This particle functions as a label, which is opposed to direct precipitation of the antigen–antibody immunocomplex (16). Agglutination reactions are based on the formation of antibody bridges between immunoglobulin G (IgG), which is bivalent, and IgM, which is a multivalent antibody, and antigen particles possessing multiple antigenic determinants. It then is possible for antibodies to react with more than one site on a single particle or to react with equivalent sites on different particles to produce a cross-linked structure. Agglutination reactions generally are used to detect antibody in specimens directed to specific antigens sensitized on particles (passive or indirect agglutination) (16). Reverse agglutination using a specified antibody sensitized on a particle surface can be used to detect soluble antigen in a sample. It should be noted that a hapten, which contains a single antigenic determinant (e.g., drug residue) would not form the cross-linked structure and could not be agglutinated unless it was immobilized on a solid surface (16).

A number of different particles have been used as solid phases for particle agglutination assays. About 100 years ago, the first direct agglutination reaction (17) was observed in a reaction involving bacteria and an infected patient serum. The reaction occurred between intrinsic antigen on the surface of the bacterial particle and its corresponding antibody. Direct agglutination is still used for immunological diagnosis of microbial infection (e.g., *Brucella* and *Salmonella*) (16).

The advancement of technology for manipulation of particles, along with the development of sophisticated methods for synthetic organic chemistry and antigen characterization, has brought about creation of particles to replace natural antigen particles. The particle would be sensitized with antigen or an antibody specific to the target in passive (or indirect) agglutination (16).

1. Hemagglutination. The principle of the passive hemagglutination test is based on the least amount of soluble antigen required to inhibit agglutination of red blood cells (Fig. 21-7). This is the concentration of antigen in the last tube that will give a wide ring agglutination pattern (known as a mat) (18).

Methods based on the agglutination are semiquantitative procedures. It is typical to use a twofold dilution scheme (Fig. 21-8). Therefore, the procedures can only yield results that reflect the dilution sequence. If the interval between antigen concentration was greater, then the inherent error also would be greater. However, the narrower the range, the more accurate the assay (19).

Hemagglutination assays are easy to perform and possess the desired sensitivity. Unfortunately, they require large quantities of antiserum, and some antibody preparations do not react with red blood cells. The biggest potential disadvantage is that there may be
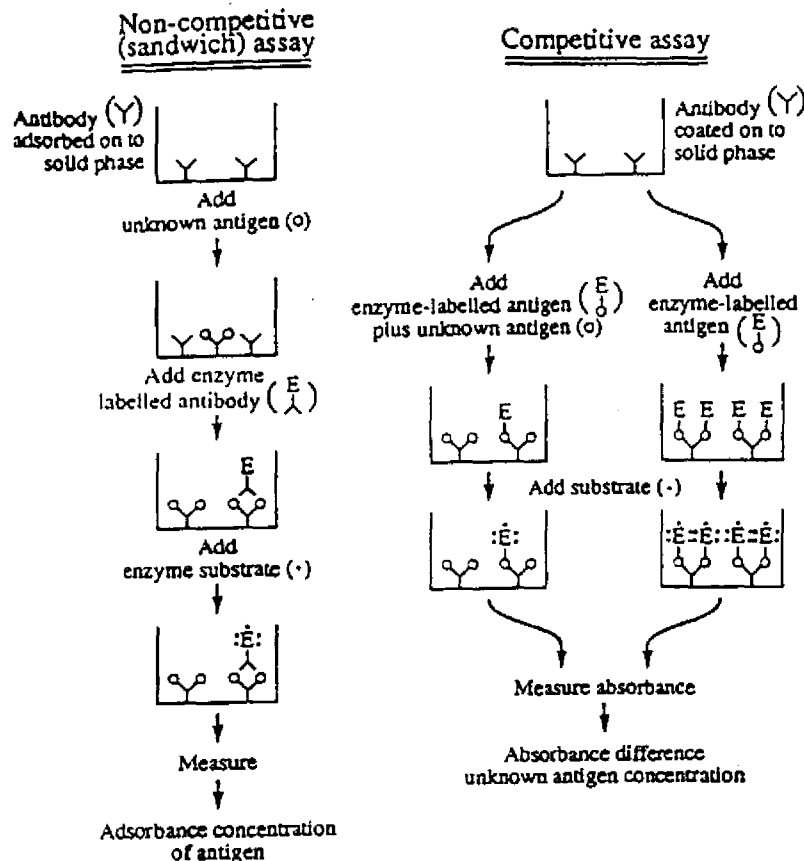
Non-competitive
(sandwich) assay

Competitive assay

nonspecific interaction from other proteins also capable of causing agglutination (20).

2. Latex Agglutination. Polystyrene latex can be used for attachment of antibodies or soluble antigens, either via adsorption or covalent attachment, in agglutination reactions. Latex particles have found wide application in agglutination immunoassays because of their stable characteristics and the ease with which antibodies and antigens can be attached chemically to their surface. Latex particles can be coated or sensitized with specific antisera and then mixed with extracts of sample to test for the presence of a specific antigen (Fig. 21-9). When the antigen molecule is a hapten, it is necessary to set up the immunoassay as an inhibition of agglutination (Fig. 21-10). Here a protein–hapten conjugate is synthesized and attached to the latex particles. Agglutination occurs when the particles are mixed with corresponding antisera (16). Addition of free hapten to the mixture will inhibit the reaction via competition for antibody sites. The concentration of analyte in the sample can be determined by comparison with standard antigen solutions (16).

21.2.2.3.2 Immunodiffusion

1. Single Radial Immunodiffusion. The polyclonal antiserum prepared against the desired antigen is uniformly dispensed in an agar gel (21). Standards of known concentration of the antigen and sample extracts are placed into the appropriate wells cut into the agar. Diffusion of the standards and samples into the agar cause formation of precipitin rings. At any given time, the diameter of the rings is proportional to the initial antigen concentrations in the wells (Fig. 21-11). Microgram quantities of antigen can be detected with the method. The method requires large quantities of antiserum and is not ideal for analysis of sparingly soluble antigens. However, gels that contain urea do enable analysis of such proteins (e.g., gluten) (22).

2. Double Diffusion. Double diffusion assays are not used quantitatively; they enable the user to obtain information about the immunochemical relationship (similarities and dissimilarities) of several antigens (23). An extract may be screened against several different antisera by placing the extract in the center well and the antisera in peripheral wells (Fig. 21-12).

Competitive assay

Antibody Particle   Antigen in Sample

B)

Antigen or Hapten   Antibody in Sample
Conjugate Particle

21-6
figure

Principles of particle agglutination immunoassay for detection of antigen with multiple epitopes (A) or antibodies (B). [From (16), used with permission.]

21-5
figure

Example of an indirect competitive enzyme-linked immunosorbent assay. [From (15), used with permission. Illustration from *Development and Application of Immunoassay for Food Analysis*, J.H. Rittenburg, Ed., copyright © 1990 by Elsevier Science Publishers Ltd., reprinted by permission of publisher.]

3. Immunoelectrophoresis. This procedure is carried out by electrophoretically separating the antigen to provide resolution of different antigenic components in the mixture prior to immunodiffusion against the antiserum (24). The antigen is electrophoresed in a gel. A trough is cut parallel to the direction of the separation. An antiserum is added to the trough and the separated antigens diffuse toward one another, which results in formation of precipitates (Fig. 21-13).

4. Rocket Immunoelectrophoresis. In this method, the antiserum is dispensed into the agarose gel (25). The antigen standards and sample extracts are added to the small wells cut into one end of the gel. The antigens then electrophorese into the antibody-containing gel. The assay is designed such that antigen migrates with a sparing to no antibody migration. The rocket-shaped

21-7
figure

Principles of particle inhibition immunoassay for detection of antigen with a single epitope, using antigen particles with free (A) or fixed (B) antibody. [From (16), used with permission.]

precipitates that form in the gel have heights that are dependent on antigen concentration (Fig. 21-14).

21.2.2.3.3 Quantitative Precipitin Techniques For quantitative precipitin techniques (26), an insoluble complex forms following interaction between a soluble antigen and specific antibodies (Fig. 21-15). This

(Qualitative Assay, Screening)

1. Drop Absorbing Diluent
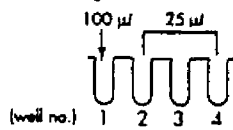
2. Add Serum Specimen

3. Make Serum Dilutions
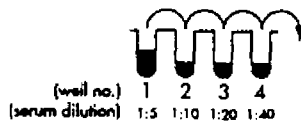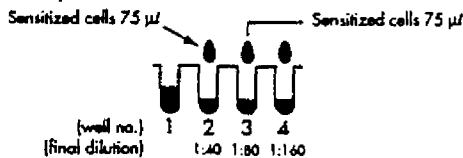
4. Drop Cells

5. Mix on a tray mixer (automatic vibrator), cover plate and incubate for 2 hours

(Qualitative Assay)

1. Drop Absorbing Diluent
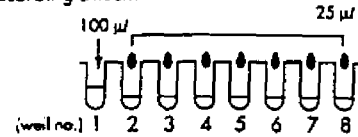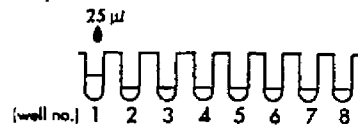
2. Add Serum Specimen

3. Make Serum Dilutions
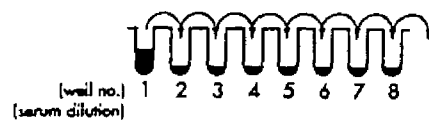
4. Drop Cells
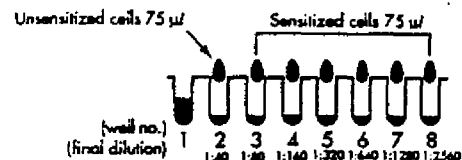
5. Mix on a tray mixer (automatic vibrator), cover plate and incubate for 2 hours

21-8 figure Hemagglutination assay for detection of antibodies to *T. pallidum* antigen based on qualitative and quantitative test protocols. This is a clinical application, but a similar format would be used for food-based application. [From (16), used with permission.]

method works in solution, not in a gel. The amount of precipitate is subsequently analyzed by protein assay. A fair amount of sensitivity is achievable, but the assay requires long times to obtain maximal precipitate formation. Generation of biphasic antigen concentration versus precipitate curves is a potential problem.

21.2.2.3.4 Immunoaffinity Columns An immunoaffinity column is constructed by attaching antibodies with specificity for certain analyte to a solid-phase support (e.g., gel matrix). A sample is extracted and passed through the column. The analyte of interest binds to the antibodies and can be eluted and quantified using fluorescent derivitization or instrumental quantification [(e.g., high performance liquid chromatography (HPLC)] (Fig. 21-16).

21.2.3 Considerations for Immunoassay Development

It is not possible, because of the scope and length of this chapter, to describe in any great detail all the considerations for development of all the immunoassays. It is appropriate, however, to provide in brief detail the

steps required to develop one such method. Since development and application of ELISAs for detection of small molecules important in the food industry are constantly on the rise, an overview of the general procedure for developing a direct competitive ELISA is provided, along with the procedure to validate such an assay.

21.2.3.1 Overview

The key tasks required for ELISA development for haptens are as follows: (1) Prepare a suitable immunogen; (2) immunize host animal (e.g., mouse or rabbit); (3) obtain test bleeds to titer antisera for specific antibodies; (4) develop an assay for optimizing (balancing) of antibodies and enzyme conjugate; (5) apply the test to the desired sample matrix; and (6) validate the method.

To elicit an immune response, the hapten must be chemically linked (in vivo and in vitro) to a carrier molecule, namely a protein. If a reactive group is not present, one must be added onto a portion of the molecule. Amino or carboxyl groups are added, which then enables the molecule to be linked to the carrier protein via an amino or carboxyl group. Commonly used car-

ANTIBODY SENSITIZED LATEX PARTICLES

+

TARGET ANTIGEN
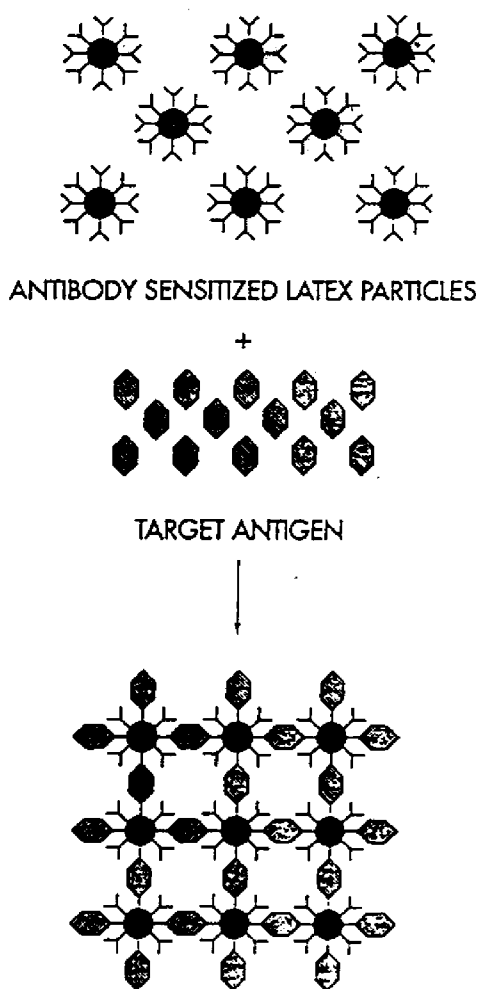
PARTICLES AGGLUTINATE-VISIBLE CLUMPING EVIDENT

21-9
figure

Illustration of a latex agglutination reaction for detecting antigen in a sample. Latex particles approximately 1.0 μm in diameter are coated with primary antibody. These particles are then mixed with the sample extract and if the target antigen is present a visible agglutination reaction will occur. [From (15), used with permission.]

SAMPLE WITH ANTIGEN | **SAMPLE WITH NO ANTIGEN**

+

+

+

|

+

POSITIVE RESULT - NO AGGLUTINATION | **NEGATIVE RESULT - LATEX AGGLUTINATION**

KEY:

Antigen coated latex   Sample containing target antigen

Primary antibody   Sample containing target antigen

21-10
figure

Illustration of a latex agglutination inhibition reaction for detecting small molecule antigens in a sample. The latex particles are coated with a preparation of the antigen and then mixed with the sample extract and the primary antibody. If the target antigen is present in the sample it will inhibit the agglutination reaction from occurring. [From (15), used with permission.]

riers are BSA, keyhole limpet hemocyanin (KLH), and bovine gamma globulin (BGG). Reports of hemisuccinate (28) and oxime (29) derivatizations appear in the literature. Periodate is a coupling method also cited (30). The mixed anhydride reaction (31), N-hydroxysuccinimide reaction (32), two-step glutaraldehyde reaction (33–35), carbodiimide (36), and diazotization (37) have been used to couple the derivatized hapten to the carrier molecule. The advantages and disadvantages of use of these methods are described in (38).

Once a titer is observed in the test serum and the reaction is shown to be specific, the reagents are optimized to generate the best possible curve in the desired

range of analyte concentration. The reagents often are first standardized in pure solutions of the hapten, and then the desired sample matrix is applied to the assay. Samples that would be analyzed for any specific analyte could be one or more of the following: tissue (e.g., liver, kidney, muscle), dairy products (e.g., milk, yogurt, and cheese), feeds, cereal grains, or processed foods.

When an assay is optimized, the antibody concentration and enzyme-labeled conjugate concentrations are set up so that the assay operates within the linear part of the curve (absorbance versus concentration of hapten). Many assays are designed to work around a concentration that is regulated. For example, aflatoxins are regulated by the United States Food and Drug Administration (FDA) at 20 ppb (39). An assay for detection of this mycotoxin, whether it is used to screen for the presence or absence of the toxin, or quantitatively to determine an actual concentration, would

a) "Identity"   b) Partial identity   c) Non-identity

21-12

figure

Double immunodiffusion methodology. (a) Line of confluence obtained with two antigens that cannot be distinguished by the antiserum used. (b) Spur formation by partially related antigens having a common determinant x but individual determinants y and z reacting with a mixture of antibodies directed against x and y. The antigen with determinants x and z can only precipitate antibodies directed to react to x. The remaining antibodies (Ab_y) cross the precipitin line to react with the antigen from the adjacent well, which has determinant y giving rise to a spur over the precipitin line. (c) Crossing over of lines formed with unrelated antigens. [From (11), used with permission. Illustration from *Development and Application of Immunoassay for Food Analysis*, J.H. Rittenburg, Ed., copyright © 1990 by Elsevier Science Publishers Ltd (original publisher). Chapman & Hall, Ltd (current publisher).]

Precipitation rings

Antibody in Agar

21-11

figure

Single radical immunodiffusion: relation of antigen concentration to size of precipitation ring formed. Antigen at the higher concentration (Ag_1) diffuses further from the well before it falls to the level giving precipitation with antibody near optimal proportions. [From (11), used with permission.]

Stage 1   Stage 2   Antigen
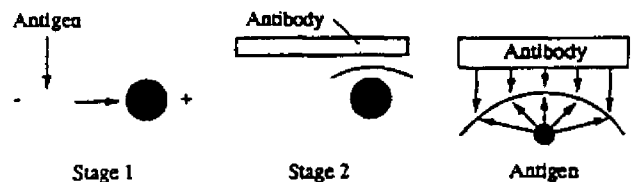
21-13

figure

The principles of immunoelectrophoresis. Stage 1: Electrophoresis of antigen in agar gel. Antigen migrates to hypothetical position shown. Stage 2: Current stopped. Trough cut in agar and filled with antibody. Precipitin arc formed. Because antigen theoretically at a point source diffuses radially, and antibody from the trough diffuses with a plane front, they meet in optimal proportions for precipition along an arc. The arc is closest to the trough at the point where antigen is in highest concentration. [From (11), used with permission.]

need to accurately detect aflatoxins above and below the 20 ppb concentration.

To construct an assay that provides the desired sensitivity, the antibody and enzyme conjugate must work together. When an assay is constructed, time is a key factor. Usually, an assay will be developed to work in the shortest time possible necessary to generate the desired color and sensitivity. Color development is extremely important when an assay is designed to be read visually. Distinct color must be observable at the control concentration as well as above and below that level.

An assay needs to be developed in a manner so that little or no color development is due to nonspecific color in the test. There are various ways antibody can be loaded onto the solid phase, and various ways plastic sites not coated with antibody can be blocked with agents to decrease or eliminate nonspecific binding of enzyme conjugate.

Optimization of an assay requires a discussion in much greater detail. Therefore, the reader is encouraged to review additional discussions on the subject (13, 14).

ELISAs can be developed using microtiter wells

(e.g., polystyrene) or membranes (e.g., nitrocellulose, nylon, polypropylene) as solid phases. The membranes are immobilized onto dipsticks, cups, or disks. Choices for enzymes include horseradish peroxidase, alkaline phosphatase, glucose oxidase, and β-galactosidase. Depending on the substrate chosen and the method used to terminate the reaction, different colors (e.g., green, blue, yellow) can be generated. Regardless of which enzyme is chosen, it should satisfy a number of

figure 21-14
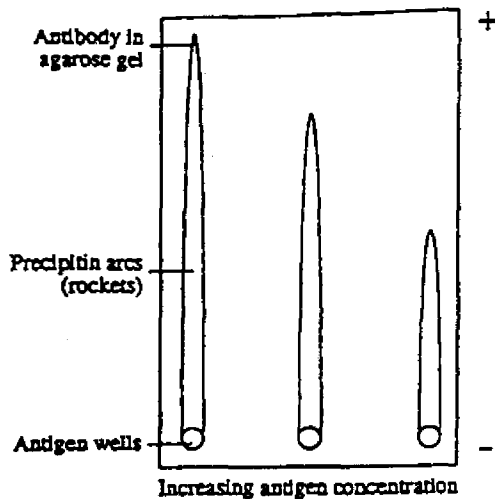
Rocket electrophoresis. Antigen, in this case human serum albumin, is electrophoresed into gel containing antibody. The distance from the starting well to the front of the rocket-shaped arc is related to antigen concentration. In the example shown, human serum albumin is present at relative concentrations from left to right: 3, 2, and 1. [From (47), used with permission.]

criteria: (1) high turnover number; (2) easily detectable product (high extinction coefficient of product in a spectral region where substrate does not absorb light, and if fluorescence detection is employed, high quantum yield of fluorescence of product); (3) long-term stability; (4) high retention of activity after coupling; (5) absence of endogenous activity in sample; (6) cost effectiveness; and (7) abundant supply (40).

21.2.3.2 Method Validation

Once optimized and applied to the desired sample(s), the method must be validated. Generally, there are nine criteria that must be satisfied to complete the validation (41). As these criteria are described below, data from an ELISA used to detect sulfamethazine in milk (42) will be used to illustrate certain criteria.

Sulfonamide residues can and do occur in milk from any of several ways. Mastitis therapy, deliberate feeding, inadvertent feeding, and the use of sulfamethazine-containing boluses to prevent infection in cows that have calved are some of the common ways for sulfonamide residues to occur in milk. Surveys and studies have been performed that showed that 50% or more of the milk samples analyzed contained measurable amounts of sulfamethazine residues (34).

21.2.3.2.1 Limit of Detection (Chemical Sensitivity)
This is the smallest quantity or concentration of an analyte that can be reliably distinguished from background in the test.

Quantify the amount of free antibody or antigen in halves of the supernatant
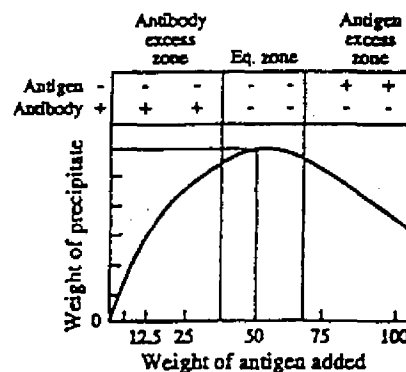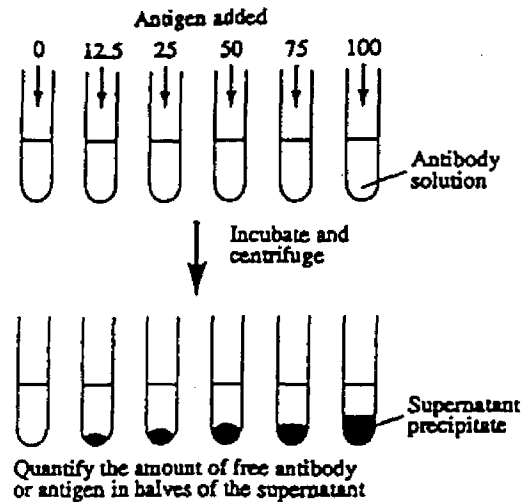
Stylized curve for a monospecific system of one antigen and the corresponding antibody

figure 21-15

Qualitative precipitin reaction. A series of tubes containing a standard quantity of antibody is prepared and to these, various amounts of antigen are added. Following incubation and centrifugation, the precipitate and supernatant are separated and the amount of antibody and antigen in equal halves of the supernatant quantified. The antibody content of the serum can be calculated from the equivalence point where no antigen or antibody is present in the supernatant. All the antigen added is therefore complexed in the precipitate with all the antibody available and the antibody content is given by subtracting the weight of antigen added from the weight of the antibody-antigen precipitated. [From (15), used with permission. Illustration from the *Development and Application of Immunoassay for Food Analysis,* J.H. Rittenburg, Ed., copyright © 1990. Elsevier Science Publishers, Ltd (original publisher). Chapman & Hall Ltd (current publisher).]

When determining the limit of detection for a direct competitive ELISA, the following equation can be used:

$$\text{Limit of detection} = \frac{X_0 - 2SD}{X_0} \times 100\% \qquad [1]$$

Activated · Monoclonal · Affinity · Antigen · · Purified
sepharose · antibody · absorbent · mixture · · antigen
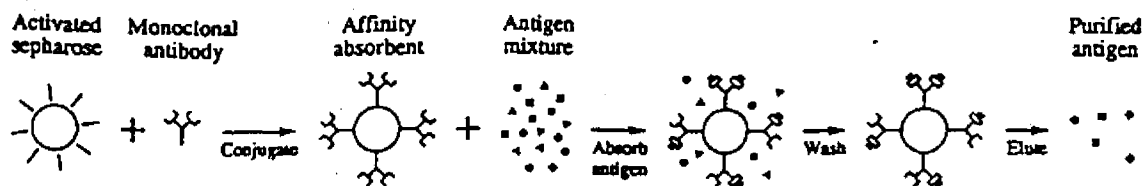
21-16
figure

Affinity chromatography. A column is filled with Sepharose-linked antibody. The antigen mixture is poured down the column. Only the antigen binds and is released, by change in pH, for example. An antigen-linked affinity column will purify antibody, obviously. [From (11), used with permission.]

where:

X_0 = mean of the absorbance value for 0 parts per billion (ppb)

2SD = 2 times the standard deviation

The mean of the 0 ppb value is the composite value obtained from the average of a number of standard curves. For example, the limit of detection for an ELISA used to detect sulfamethazine in milk is 0.7 ppb (Table 21-1). The test enables the detection of sulfamethazine well below the safe level set by the FDA (43).

21.2.3.2.2 Crossreactivity (Chemical Specificity) This is the extent to which the assay responds to only the specified analyte and not to other compounds or substances in the sample.

The sulfamethazine antibody was found to be highly specific for sulfamethazine and did not bind to closely related sulfonamides (Table 21-2). There was 14% crossreactivity with sulfamerazine, which is not

surprising since sulfamerazine only lacks one methyl group that sulfamethazine possesses. The antibody did not recognize penicillin or chlortetracycline, which are commonly used in combination with sulfonamides.

21.2.3.2.3 Reproducibility This is the ability of the assay to duplicate results in repeat determinations. It is the opposite of variability in the assay.

1. **Intra-Assay Variability.** This is the variability between replicate determinations in the same assay. Intra-assay variability for the sulfamethazine ELISA ranged from 6.02% to 7.70%, with overall variability being 6.89%. This assay is highly repeatable.

2. **Inter-Assay Variability.** This is the variability between replicate determinations from different groups. Inter-assay variability for the sulfamethazine ELISA ranged from 2.84% to 17.44%, with an overall value of 8.54%. A coefficient of variation (CV) below 10% is very good. Chemical methods often have CVs of ±50% when measurements are taken in the ppb range (44).

21-1
table

Limit of Detection for the Direct Competitive ELISA for Sulfamethazine in Milk

CONCENTRATION OF SULFAMETHAZINE (ppb) Absorbance of 650 nm						
0	2.5	5	10	25	50	
0.735	0.620	0.544	0.367	0.209	0.122	
0.774	0.614	0.540	0.377	0.205	0.124	
0.720	0.568	0.521	0.347	0.196	0.117	
0.724	0.560	0.493	0.352	0.192	0.137	
0.704	0.560	0.467	0.364	0.215	0.137	
0.734	0.594	0.510	0.421	0.239	0.174	
0.732	0.586	0.513	0.371	0.209	0.135	Mean

$$\text{Limit of detection} = \frac{X_0 - 2SD}{X_0} \times 100$$

$$= \frac{0.732 - 2(0.0215)}{0.732} \times 100$$

$$= 94.1\%$$

94.1% absorbance corresponds to 0.7 ppb concentration of sulfamethazine when graphed on logit-log paper.

From (42), used with permission.

21-2
table

Percent Relative Crossreactivity of Anti-Sulfamethazine Polyclonal Antibody Determined Using Sulfamethazine and Other Drugs

Compound	CONCENTRATION REQUIRED TO INHIBIT 50% OF ANTIBODY	
	Binding (ppb)	Percent Crossreactivity[1]
Sulfamethazine	90	100
Sulfamerazine	630	14
Sulfathiazole	>50,000	<0.18
Sulfapyridine	>50,000	<0.18
Sulfaquinoxaline	>50,000	<0.18
Sulfachloropyridazine	>50,000	<0.18
Sulfadimethoxine	>50,000	<0.18
Sulfanilamide	>50,000	<0.18
Chlorotetracycline HCl	>50,000	<0.18
Procaine penicillin	>50,000	<0.18
p-Aminobenzoic acid	>50,000	<0.18

From (42), used with permission
[1]Relative percent crossreactivity =

$$\frac{\text{Concentration of sulfamethazine required to inhibit 50\% of antibody binding (ppb)}}{\text{Concentration of other antimicrobial agent required to inhibit 50\% of antibody binding (ppb)}} \times 100$$

21.2.3.2.4 Reference Correlation This is the degree of closeness of the linear relationship between the results obtained using the ELISA and a reference assay [(e.g., HPLC or thin-layer chromatography (TLC) versus ELISA)] over the range of the test.

When 64 milk samples were analyzed for sulfamethazine by ELISA and HPLC, a correlation of 94.9% was obtained. Excellent agreement between immunoassays and chemical methods is possible. The calculation of reference correlation is performed during collaborative studies carried out by AOAC International (45).

21.2.3.2.5 Sensitivity This is the ability of the test to detect positive samples as positive. It is the percent positivity in a population of true positives.

When 74 raw milk samples were analyzed for sulfamethazine by ELISA, the sensitivity was determined to be 20/21 = 95.2% (>10 ppb sulfamethazine is positive).

21.2.3.2.6 Specificity This is the ability of the test to detect negative samples as negative. It is the percent test negativity in a population of true negatives.

When 74 raw milk samples were analyzed for sulfamethazine by ELISA, the specificity was determined to be 52/53 = 98.1% (<10 ppb sulfamethazine is negative) (42).

21.2.3.2.7 Overall Accuracy This is the combined or total ability of the test to correctly detect positive and negative samples (sensitivity and specificity = overall accuracy).

The overall accuracy for the sulfamethazine ELISA was determined to be 20/21 + 52/53 = 72/74 = 97.3%, which demonstrates that ELISAs can produce highly accurate results.

21.2.3.2.8 Stability This is the usable shelf life of the kit for specified storage conditions. It can be determined by accelerated aging studies and confirmed by real-time testing. This validation point, is, of course, important if a method is to be commercialized into a test kit format (46). Many ELISAs that are commercially available in kits are stable for 6 months to 1 year when stored at 4°C. Some tests can be stored at room temperature.

21.2.3.2.9 Ruggedness It is common for ruggedness testing to be included in the validation. An example of ruggedness testing for an ELISA would be to determine test performance over a range of temperatures (e.g., extreme temperatures). This might be a concern when a test is used in the field in a setting exposed to seasonal temperature fluctuations (e.g., slaughterhouse).

21.3 APPLICATIONS

Immunoassays and particularly ELISAs are finding increasing application every year for food analysis and surveillance testing. A chapter of this nature cannot even begin to summarize, let alone discuss in any great detail, each type of immunoassay and how it is being used. There are numerous books and lengthy review articles that describe the drawbacks to using chemical methods (e.g., thin-layer chromatography, high performance liquid chromatography, gas chromatography–mass spectroscopy) as well as older immunoassay (e.g., gel diffusion, agglutination) for routine sample analysis (15, 47–50). These articles and books also describe in great detail the development and application of ELISAs for analysis of foods, whether the samples comprise food components, are naturally occurring contaminants, or are by-products of crop or livestock production. The literature describes the potential interferences in samples and discusses which samples are more difficult to analyze.

The references cited earlier discuss not only assay development and application but also issues concerning use of ELISAs by regulatory agencies for sample analysis. There also are discussions concerning implementation of ELISAs for uses other than research purposes (e.g., quality control testing, routine sample

analysis, regulatory testing, field versus laboratory setting).

Also provided in the references are lists of tests that can be purchased commercially for testing various commodities. Integration of ELISA kits and other immunoassays into routine testing programs is an ever-increasing occurrence. In many instances, immunoassays are used for initial screening, and then classical microbiological tests are used for confirmation. There are more and more immunoassays to choose from that enable the user to obtain quick and accurate results for a modest price. This information can be gathered from numerous web sites. Several companies that market immunoassays for food testing include Neogen Corporation, Lansing, MI (*http://www.neogen.com/*), Indexx (*http://www.idexx.com/*), and Vicam (*http://www.vicam.com/VICAM/*).

Whether an individual is a student in the area of food science and technology, or an experienced industrial researcher or academician, information can be gained from memberships in a number of associations, such as AOAC International and the Institute of Food Technologists (IFT). A comprehensive list of commercial immunoassay kits for detection of food pathogens (e.g., *Salmonella* and *E. coli*), mycotoxins, and numerous analytes is obtainable from the AOAC International web site (*http://www.aoac.org*). The AOAC International web site is hyperlinked to many other organizations [e.g., American Association of Cereal Chemists (AACC) (*http://www.scsoc.org/aacc/*)] and to government agencies such as the FDA and the Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture. By visiting these sites one can learn about all the recent regulations put in place for testing meat, poultry, eggs, and seafood for *E. coli* and *Salmonella*, for which immunoassays can be applied. One also can view information that consumers can access from the FDA web site that describes the risks of foodborne illness from foods, as well as precautions that can be taken to make smart buying choices, and the proper types of food handling and preparation to avoid or decrease risk of illness from foodborne pathogens.

The IFT website (*http://www.ift.org*) includes information about allergen testing in foods. Immunoassays can be used for detection of food allergens (51, 52) to ensure that they are properly controlled in food-processing plants. Using hazard analysis critical control point (HACCP) type flow diagrams, it is possible to identify points for control of allergens in the process (51). The sensitivity, specificity, and speed of use of immunoassays enables real-time testing to be done to verify that a system is allergen-free. Radioimmunoassay (53–55) and ELISA (52, 56) methods are being developed, but are not yet validated methods, to ensure that a system is allergen-clean. This is a good example of an area where the value of immunoassay

has been observed, method development is occurring, which will lead to validation of the method. The application of immunoassays, and ELISAs, in particular, continue to grow in number and scope (57–63). Their use will continue to be exploited for years to come and will serve as tools for both the research and industrial communities.

21.4 SUMMARY

It is obvious upon review of this chapter that a great number of immunoassays exist that can be used for analysis of large and small molecules. All have advantages and potential disadvantages that must be considered when selecting a method for analysis. Numerous factors also must be considered in developing and validating any immunoassay method.

In an era when questions are raised on a regular basis that focus on the safety of the food supply, when consumers are aware and concerned about the food they are buying, when large numbers of samples need to be tested on a routine basis, the need is great and the application of immunoassay, mainly ELISAs, is timely. While immunoassays were once a tool used only for analysis of clinical samples, development and use of immunoassays for detection of food constituents, additives, natural contaminants, growth promoters, and the like are becoming more and more commonplace in the testing scenario. With the advent of commercial kits, testing can be handled efficiently and effectively for a minimal cost. It is an area of explosive growth, with newer, more sensitive, and more specific methods available for sample analysis. Consumers can rest assured that implementation of immunological testing tools will monitor the safety of their food supply in a more effective manner than has every before been possible. With Internet access both consumers and scientists can gather information that before was unavailable or took a long time to obtain. Information technology and immunoassays together have brought the area of food analysis into the modern age.

21.5 STUDY QUESTIONS

1. We live in an era when consumers are becoming more concerned about food safety. Where might a consumer go to learn about the programs or regulations that are in place for testing food commodities? What government agencies or scientific associations might be able to provide this type of information and can this information be accessed by the consumer? How?

2. An ELISA was developed for use by a research group for routine screening of aflatoxin B_1 (AFB_1) in peanut butter. The reagents (the anti-AFB_1 antibody and the enzyme-labeled AFB_1 conjugate), were commercially purchased.

Aflatoxin B_1 was extracted from the peanut butter using organic solvent. According to the procedure, the AFB_1 antibodies were previously attached to the wells in the microtiter plate. Then the sample extract (with an unknown amount of AFB_1) was mixed with the enzyme-labeled AFB_1. This mixture was added to one set (set #1) of duplicate wells with anti-AFB_1 antibodies attached to the microtiter wells. To another set of wells (set #2) (with antibodies already coated onto the wells) was added enzyme-labeled AFB_1 mixed with an equal volume of solvent. The plates were incubated for 10 min at room temperature. After the reactants were washed from the microtiter wells, the substrate of the enzyme was added to all the wells. The microtiter wells contents were incubated for 10 min at room temperature. A stopping reagent was added to the wells to inhibit further enzyme activity and the absorbance was read spectrophotometrically (with an ELISA reader) at the appropriate wavelength [e.g., 405 nm for the substrate ABTS-H_2O_2; ABTS = 2,2'-azino-d-(3-ethyl-benzthiazoline sulfonate)].

a. Draw a schematic diagram of the assay components in each of the duplicate sets of wells, for set #1 and for set #2. Be sure to label the components with anti-B_1 antibody, enzyme-labeled AFB_1, sample or unknown AFB_1 concentration, blank solvent, substrate, stopping reagent.

b. Select the correct answer for each question:
  1. Is this type of ELISA assay competitive direct, competitive indirect, or sandwich ELISA?
  2. Would the absorbance at 405 nm of the well contents where AFB_1 enzyme labeled conjugate and blank solvent were incubated be high, low, or have no absorbance?
  3. Would a very low absorbance reading at 405 nm suggest that the amount of AFB_1 in the peanut butter sample is low, high, or there is none present?

3. Describe how an immunoaffinity column could be used to purify a substance from a food extract. Are there any commercially available immunoaffinity columns? How would you find out which one(s) are certified or approved for use in the food testing industry? How would you use the Internet to find the desired information?

4. List the key tasks required for development of an ELISA for detection of a low-molecular-weight compound.

5. Describe the nine criteria required to validate a method.

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Application of Enzymes in Food Analysis

Joseph R. Powers

22.1 INTRODUCTION

Enzymes are protein catalysts that are capable of very great specificity and reactivity under physiological conditions. Enzymatic analysis is the measurement of compounds with the aid of added enzymes or the measurement of endogenous enzyme activity to give an indication of the state of a biological system including foods. The fact that enzyme catalysis can take place under relatively mild conditions allows for measurement of relatively unstable compounds not amenable to some other techniques. In addition, the specificity of enzyme reactions can allow for measurement of components of complex mixtures without the time and expense of complicated chromatographic separation techniques.

There are several uses of enzyme analyses in food science and technology. In several instances, enzyme activity is a useful measure for adequate processing of a food product. The thermal stability of enzymes has been used extensively as a measure of heat treatment; for example, peroxidase activity is used as a measure of adequacy of blanching of vegetable products. Enzyme activity assays are also used by the food technologist to assess potency of enzyme preparations used as processing aids.

The food scientist can also use commercially available enzyme preparations to measure constituents of foods that are enzyme substrates. For example, glucose content can be determined in a complex food matrix containing other monosaccharides by using readily available enzymes. A corollary use of commercially available enzymes is to measure enzyme activity as a function of enzyme inhibitor content in a food. Organophosphate insecticides are potent inhibitors of the enzyme acetylcholinesterase and hence the activity of this enzyme in the presence of a food extract is a measure of organophosphate insecticide concentration in the food. Also of interest is the measurement of enzyme activity associated with food quality. For example, catalase activity is markedly increased in milk from mastitic udders. Catalase activity also parallels the bacterial count in milk. Another use of enzyme assays to determine food quality is estimation of protein nutritive value by monitoring the activity of added proteases on food protein samples (see Chapter 17). Enzymes can be used to measure the appearance of degradation products such as trimethylamine in fish during storage. Enzymes are also used as preparative tools in food analysis. Examples include the use of amylases and proteases in fiber analysis (Chapter 12) and the enzymatic hydrolysis of thiamine phosphate esters in vitamin analysis.

To successfully carry out enzyme analyses in foods, an understanding of certain basic principles of

enzymology is necessary. After a brief overview of these principles, examples of the use of enzymatic analyses in food systems are examined.

22.2 PRINCIPLES

22.2.1 Enzyme Kinetics

22.2.1.1 Overview

Enzymes are biological catalysts that are proteins. A catalyst increases the rate (velocity) of a thermodynamically possible reaction. The enzyme does not modify the equilibrium constant of the reaction, and the enzyme catalyst is not consumed in the reaction. Because enzymes affect rates (velocities) of reactions, some knowledge of enzyme kinetics (study of rates) is needed for the food scientist to effectively use enzymes in analysis. To measure the rate of an enzyme-catalyzed reaction, typically one mixes the enzyme with the substrate under specified conditions (pH, temperature, ionic strength, etc.) and follows the reaction by measuring the amount of product that appears or by measuring the disappearance of substrate. Consider the following as a simple representation of an enzyme-catalyzed reaction:

$$S + E \rightleftharpoons ES \rightarrow P + E \qquad [1]$$
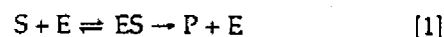
where:

S = substrate
E = enzyme
ES = enzyme–substrate complex
P = product

The time course of an enzyme catalyzed reaction is illustrated in Fig. 22-1. The formation of the enzyme substrate complex is very rapid and is not normally seen in the laboratory. The brief time in which the enzyme–substrate complex is initially formed is on the millisecond scale and is called the pre-steady-state period. The slope of the linear portion of the curve following the pre-steady-state period gives us the initial velocity (v_0). After the pre-steady-state period, a steady-state period exists in which the concentration of the enzyme–substrate complex is constant. A time course needs to be established experimentally by using a series of points or a continuous assay to establish the appropriate time frame for the measurement of the initial velocity.

The rate of an enzyme-catalyzed reaction depends on the concentration of the enzyme and also depends on the substrate concentration. With a fixed enzyme concentration, increasing substrate concentration will
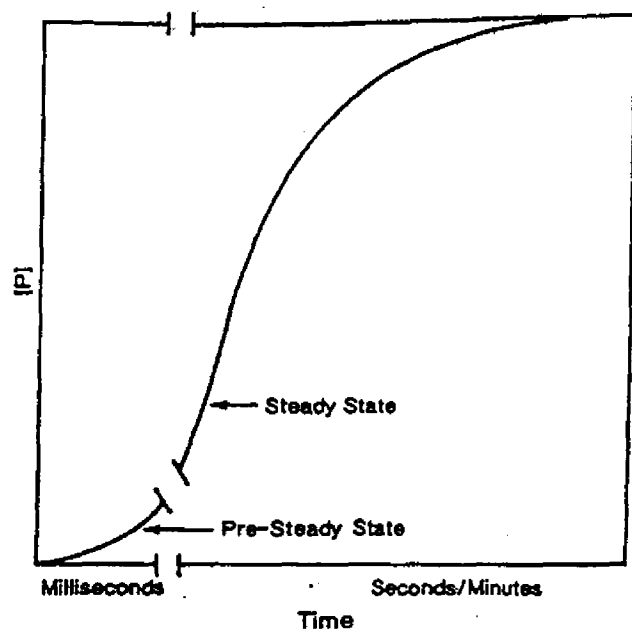
22-1 figure Time course for a typical enzyme-catalyzed reaction showing the pre-steady-state and steady state periods. [P] = product concentration.

result in an increased velocity (see Fig. 22-2). As substrate concentration increases further, the increase in velocity slows until, with a very large concentration of substrate, no further increase in velocity is noted. The velocity of the reaction at this very large substrate concentration is the maximum velocity (V_m) of the reaction under the conditions of that particular assay. The substrate concentration at which one half V_m is observed is defined as the Michaelis constant or K_m. K_m is an important characteristic of an enzyme. It is an indication of the relative binding affinity of the enzyme for a particular substrate. The lower the K_m, the greater the affinity of the enzyme for the substrate.

If we examine relationships that hold in the steady state period, the Michaelis–Menten equation can be derived for the simplified enzyme-catalyzed reaction:

$$E + S \underset{k_{-1}}{\overset{k_1 \quad k_2}{\rightleftharpoons}} ES \rightarrow E + P \qquad [2]$$
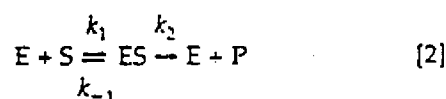
where:

k_1, k_{-1}, k_2 = reaction rate constants for reactions indicated

In the steady state, the rate of change in enzyme substrate complex concentration is zero: $d\,ES/dt = 0$ and:

Rate of disappearance of $ES = k_{-1}$ [ES] + k_2[ES] [3]

Rate of appearance of $ES = k_1$ [E][S] [4]

Then k_1 [E][S] = k_{-1}[ES] + k_2[ES] [5]

$[E_0] = [E] + [ES]$ [6]

where:

E_0 = total enzyme
E = free enzyme
ES = enzyme–substrate complex

Substituting

$[E] = [E_0] - [ES]$ [7]

$k_1([E_0] - [ES])[S] = k_{-1}[ES] + k_2[ES]$ [8]
$= (k_{-1} + k_2)[ES]$

Rearranging and solving for [ES]:

$$ES = \frac{k_1 [E_0][S]}{k_1 [S] + (k_{-1} + k_2)} \qquad [9]$$

$$= \frac{[E_0][S]}{\frac{k_{-1} + k_2}{k_1} + [S]}$$

If the collection of rate constants in the denominator is defined as the Michaelis constant (K_m):

$$K_m = \frac{k_{-1} + k_2}{k_1} \qquad [10]$$

Note that K_m is *not* affected by enzyme or substrate concentration.
Then:

$$[ES] = \frac{[E_0][S]}{K_m + [S]} \qquad [11]$$

If we define the velocity (v_0) of the enzyme-catalyzed reaction as:

$$v_0 = k_2 [ES] \qquad [12]$$

Then:

$$v_0 = \frac{k_2 [E_0][S]}{K_m + [S]} \qquad [13]$$

When all the enzyme is saturated—all substrate binding sites on the enzyme are occupied—at the large substrate concentrations in Fig. 22-2 we have maximum velocity, V_m: All of E_0 is in the ES form and

$k_2 [ES] = k_2 [E_0]$ at [S] >> K_m

and:

$$v_0 = \frac{V_m [S]}{K_m + [S]} \qquad [14]$$
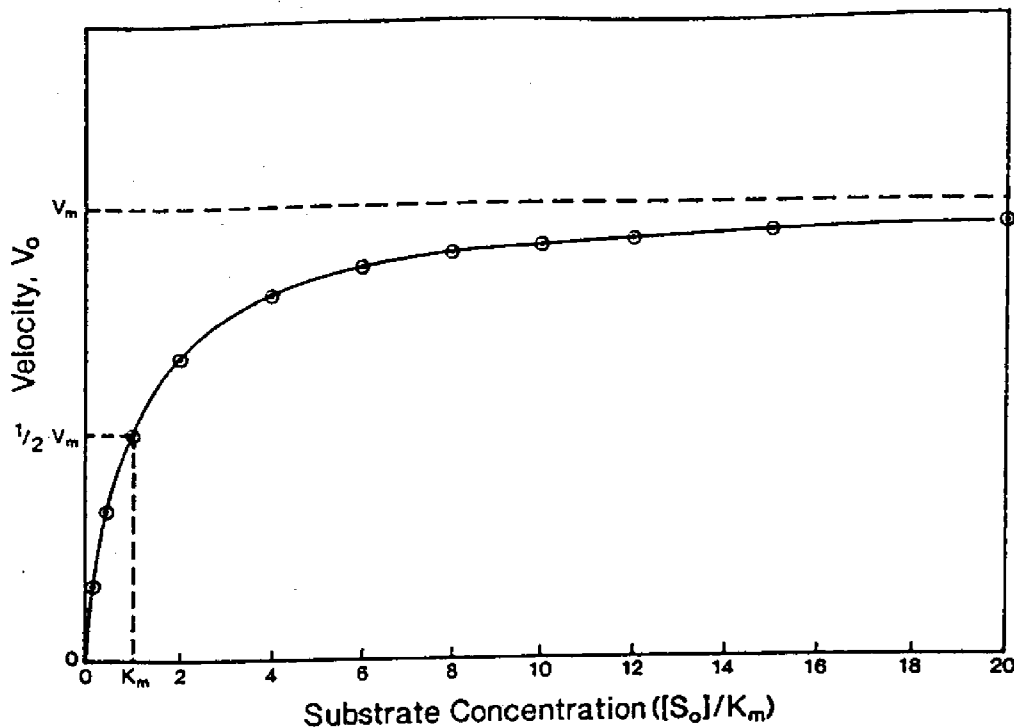
Effect of substrate concentration on the rate of an enzyme-catalyzed reaction. Plotted according to the Michaelis–Menten equation.

This is the Michaelis–Menten equation, the equation for a right hyperbola; the data plotted in Fig. 22-2 fit such an equation. A convenient way to verify this equation is to simply remember that $v_0 = 1/2\ V_m$ when [S] = K_m. Therefore, by simple substitution

$$1/2\ V_m = \frac{V_m K_m}{K_m + K_m} = \frac{V_m}{2} \qquad [15]$$

22.2.1.2 Order of Reactions

The velocity of an enzyme-catalyzed reaction increases as substrate concentration increases (see Fig. 22-2). A first-order reaction with respect to substrate concentration is obeyed in the region of the curve where substrate concentration is small ([S] $\ll$ K_m). This means that the velocity of the reaction is directly proportional to the substrate concentration in this region. When the substrate concentration is further increased, the velocity of the reaction no longer increases linearly, and the reaction is mixed order. This is seen in the figure as the curvilinear portion of the plot. If substrate concentration is increased further, the velocity asymptotically approaches the maximum velocity (V_m). In this linear, nearly zero slope portion of the plot, the velocity is independent of substrate concentration. However, note that at large substrate concentrations ([S] $\gg$ K_m), the velocity is directly proportional to enzyme concentration ($V_m = k_2$ [E$_0$]). Thus, in this portion of the curve

where [S] $\gg K_m$, the rate of the reaction is zero order with respect to substrate concentration (is independent of substrate concentration) but first order with respect to enzyme concentration.

If we are interested in measuring the amount of enzyme in a reaction mixture, we should, if possible, work at substrate concentrations so that the observed velocity approximates V_m. At these substrate concentrations, enzyme is directly rate limiting to the observed velocity. Conversely, if we are interested in measuring substrate concentration by measuring initial velocity, we must be at substrate concentrations less than K_m in order to have a rate directly proportional to substrate concentration.

22.2.1.3 Determination of Michaelis Constant (k_m) and V_m

To properly design an experiment in which velocity is zero order with respect to substrate and first order with respect to enzyme concentration, or conversely an experiment in which we would like to measure rates that are directly proportion to substrate concentration, we must know the K_m. The most popular method for determining K_m is the use of a Lineweaver–Burk plot. The reciprocal of the Michaelis–Menten equation is:

$$\frac{1}{v_0} = \frac{K_m}{V_m [S]} + \frac{1}{V_m} \qquad [16]$$

This equation is that of a straight line $y = mx + b$ where m = slope and b = y-intercept. A plot of substrate concentration versus initial velocity as shown in Fig. 22-2 can be transformed to a linear form via use of the reciprocal Equation [16] and Fig. 22-3 (Lineweaver-Burk plot) results. The intercept of the plotted data on the y (vertical) axis is $1/V_m$ while the intercept on the x (horizontal) axis is $-1/K_m$. The slope of the line is K_m/V_m. Consequently, both K_m and V_m can be obtained using this method.

A disadvantage of the Lineweaver-Burk plot is that the data with the inherently largest error, collected at very low substrate concentrations and consequently low rates, tends to direct the drawing of a best fit line. An alternative method of plotting the data is the Eadie-Hofstee method. The Michaelis-Menten equation can be rearranged to give:

$$v_0 = V_m - \frac{v_0 K_m}{[S]} \qquad [17]$$

Equation [17] is also the equation of a straight line and when v_0 versus $v_0/[S]$ are plotted, the slope of the line is $-K_m$, the y-intercept is V_m and the x-intercept is V_m/K_m. A more even spacing of the data is achieved by this method than by a the Lineweaver-Burk plot.

22.2.2 Factors That Affect Enzyme Reaction Rate

The velocity of an enzyme-catalyzed reaction is affected by a number of factors, including enzyme and substrate concentrations, temperature, pH, ionic strength, and the presence of inhibitors and activators.

22.2.2.1 Effect of Enzyme Concentration

The velocity of an enzyme-catalyzed reaction will depend on the enzyme concentration in the reaction mixture. The expected relationship between enzyme activity and enzyme concentration is shown in Fig. 22-4. Doubling the enzyme concentration will double the rate of the reaction. If possible, determination of enzyme activity should be done at concentrations of substrate much greater than K_m. Under these conditions, a zero-order dependence of the rate with respect to substrate concentration and a first-order relationship between rate and enzyme concentration exist. It is critical that the substrate concentration is saturating during the entire period the reaction mixture is sampled and the amount measured of product formed or substrate disappearing is linear over the period during which the reaction is sampled. The activity of the enzyme is obtained as the slope of the linear part of the line of a plot of product or substrate concentration versus time.

If a large number of samples are to be assayed, a single aliquot is often taken at a single time. This can be risky and will give good results only if the time at which the sample is taken falls on the linear portion of a plot of substrate concentration or product concentration versus time of reaction (see Fig. 22-5). The plot becomes nonlinear if the substrate concentration falls below the concentration needed to saturate the enzyme, if the
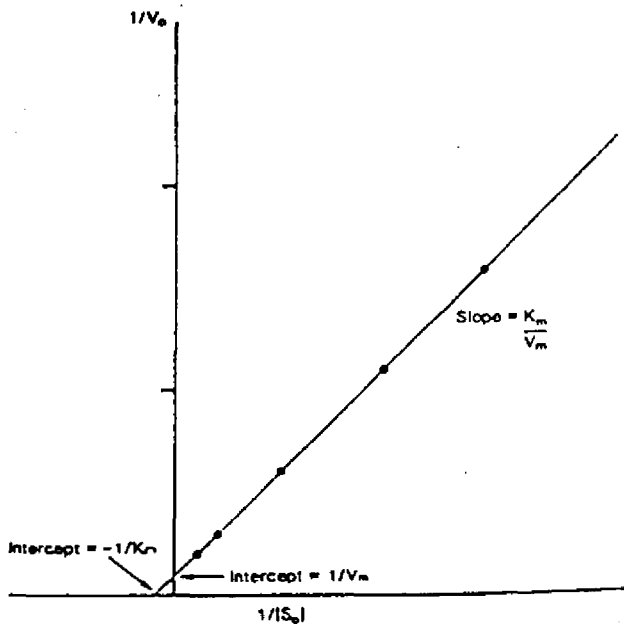
22-3 Plot of substrate–velocity data by the Lineweaver–Burk method.
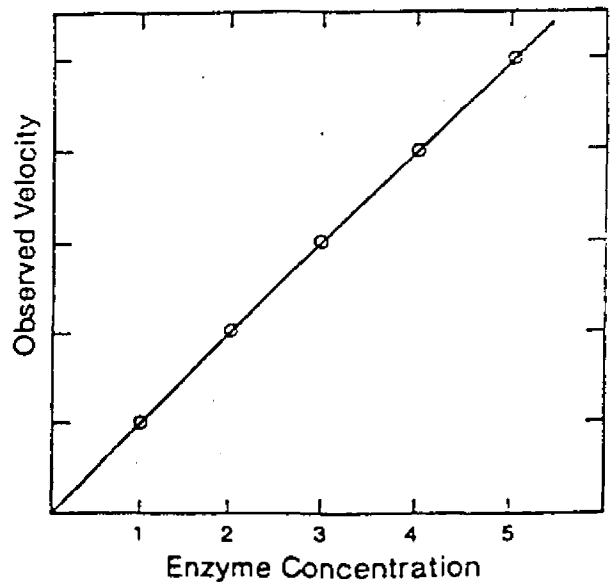
22-4 Expected effect of enzyme concentration on observed velocity of an enzyme-catalyzed reaction.
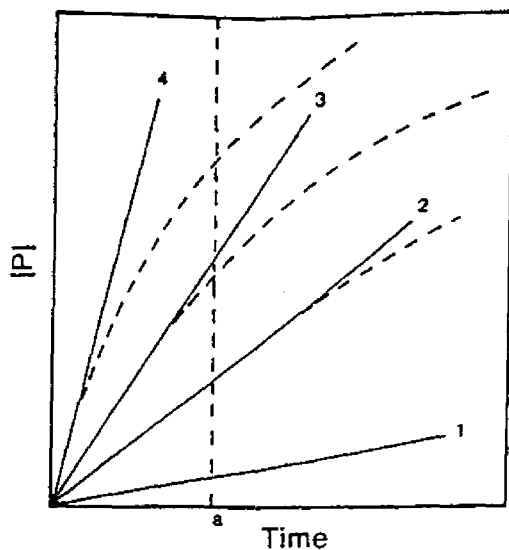
22-5 figure

Effect of enzyme concentration on time course of an enzyme-catalyzed reaction. The dashed lines are experimentally determined data with enzyme concentration increasing from 1 to 4. The solid lines are tangents drawn from the initial slopes of the experimental data. If a single time point, a, is used for data collection, a large difference between actual data collected and that predicted from initial rates is seen.

22-6 figure

Product concentration [P] formed as a function of time for an enzyme-catalyzed reaction. Line 1 is linear indicating a zero-order reaction with respect to substrate concentration [S]. The slope of line 1 is directly related to enzyme concentration. Line 2 is nonlinear. A replot of line 2 data, plotting $\log[S_0]/[S]$ versus time is linear (insert), indicating the reaction is first order with respect to substrate concentration. The slope of the replot is directly related to enzyme concentration.

increase in concentration of product produces a significant amount of back reaction, or if the enzyme loses activity during the time of the assay. Normally, one designs an experiment in which enzyme concentration is estimated such that no more than 5–10% of the substrate has been converted to product within the time used for measuring the initial rate. In the example shown in Fig. 22-5, by sampling at the single point, a, an underestimation of the rate is made for curves 3 and 4. A better method of estimating rates is to measure initial rates of the reactions, in which the change in substrate or product concentration is determined at times as close as possible to time zero. This is shown in Fig. 22-5 by the solid lines drawn tangent to the slopes of the initial parts of the curves. The slope of the tangent line gives the initial rate.

Sometimes it is not possible to carry out enzyme assays at $[S] \gg K_m$. The substrate may be very expensive or relatively insoluble or K_m may be large (i.e., $K_m > 100$ mM). Enzyme concentration can also be estimated at substrate concentrations much less than K_m. When substrate concentration is much less than K_m, the substrate term in the denominator of the Michaelis–Menten equation can be ignored and $v = (V_m[S])/K_m$ which is the equation for a first-order reaction with respect to substrate concentration. Under these conditions, a plot of product concentration versus time gives a nonlinear plot (Fig. 22-6). A plot of log $([S_0]/[S])$ versus time gives a straight line relationship (Fig. 22-6,

inset). The slope of the line of the log plot is directly related to the enzyme concentration. When the slope of a series of these log plots is further plotted as a function of enzyme concentration, a straight line relationship should result. If possible, the reaction should be followed continuously or aliquots removed at frequent time intervals and the reaction allowed to proceed to greater than 10% of the total reaction.

22.2.2.2 Effect of Substrate Concentration

The substrate concentration–velocity relationship for an enzyme-catalyzed reaction in which enzyme concentration is constant is shown in Fig. 22-2. As noted before, the rate of the reaction is first order with respect to substrate concentration when $[S] \ll K_m$. At $[S] \gg K_m$, the reaction is zero order with respect to substrate concentration and first order with respect to $[E]$. At substrate concentrations between the first-order and zero-order regions, the enzyme-catalyzed reaction is mixed order with respect to substrate concentration. However, when initial rates are obtained, a linear relationship between v_0 and E_0 should be seen.

22.2.2.3 Environmental Effects

22.2.2.3.1 Effect of Temperature on Enzyme Activity
Temperature can affect observed enzyme activity in

several ways. Most obvious is that temperature can affect the stability of enzyme and also the rate of the enzyme-catalyzed reaction. Other factors in enzyme-catalyzed reactions that may be considered include the effect of temperature on the solubility of gases that are either products or substrates of the observed reaction and the effect of temperature on pH of the system. A good example of the latter is the common buffering species Tris (tris [hydroxymethyl]aminomethane), for which the pK_a changes 0.031 per 1°C change.

Temperature affects both the stability and the activity of the enzyme, as shown in Fig. 22-7. At relatively low temperatures, the enzyme is stable. However, at higher temperatures, denaturation dominates, and a markedly reduced enzyme activity represented by the negative slope portion of line 2 is observed. Line 1 of Fig. 22-7 shows the effect of temperature on the velocity of the enzyme-catalyzed reaction. The velocity is expected to increase as the temperature is increased. As shown by line 1, the velocity approximately doubles for every 10°C rise in temperature. The net effect of increasing temperature on the rate of conversion of substrate to product (line 1) and on the rate of the denaturation of enzyme (line 3) is line 2 of Fig. 22-7. The temperature optimum of the enzyme is at the maximum point of line 2. The temperature optimum is not a unique characteristic of the enzyme. The optimum applies instead to the entire system because type of substrate, pH, salt concentration, substrate concentra-

tion, and time of reaction can affect the observed optimum. For this reason, investigators should fully describe a system in which the effects of temperature on observed enzyme activity are reported.

The data of line 2 of Fig. 22-7 can be plotted according to the Arrhenius equation:

$$k = Ae^{-E_a/RT} \qquad [18]$$

which can be written:

$$\log k = \log A - \frac{E_a}{2.3RT} \qquad [19]$$

where:

k = a specific rate constant at some temperature, $T(K)$

E_a = activation energy, the minimum amount of energy a reactant molecule must have to be converted to product

R = gas constant

A = a frequency factor (preexponential factor)

The positive slope on the left side (high temperature) of the Arrhenius plot (Fig. 22-8) gives a measure of the activation energy (E_a) for the denaturation of the enzyme. Note that a small change in temperature has a very large effect on the rate of denaturation. The slope on the right side of Fig. 22-8 gives a measure of the E_a for the transformation of substrate to product catalyzed by the enzyme. If the experiment is carried out under conditions in which V_m is measured ([S] >> K_m), then the activation energy observed will be for the catalytic step of the reaction.
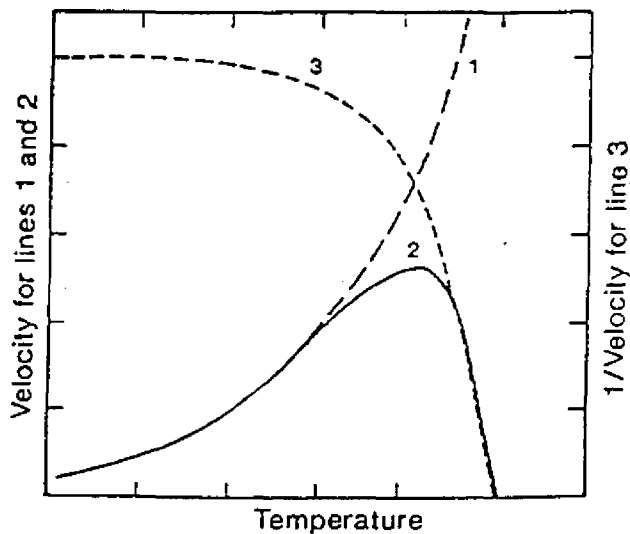
Effect of temperature on velocity of an enzyme-catalyzed reaction. Temperature effect on substrate to product conversion is shown by line 1. Line 3 shows effect of temperature on rate of enzyme denaturation (right-hand y-axis is for line 3). The net effect of temperature on the observed velocity is given by line 2 and the temperature optimum is at the maximum of line 2.
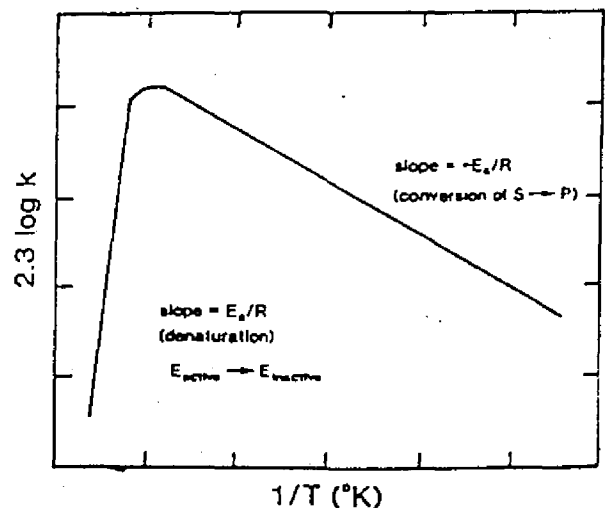
Effect of temperature on rate constant of an enzyme-catalyzed reaction. The data are plotted 2.3 log k versus $1/T$ (K) according to the Arrhenius equation, $k = Ae^{-E_a/RT}$.

22.2.2.3.2 Effect of pH on Enzyme Activity The observed rate of an enzyme-catalyzed reaction is greatly affected by the pH of the medium. Enzymes have pH optima and commonly have bell-shaped curves for activity versus pH (Fig. 22-9). This pH effect is a manifestation of the effects of pH on enzyme stability and on rate of substrate to product conversion and may also be due to changes in ionization of substrate.

The rate of substrate to product conversion is affected by pH because pH may affect binding of substrate to enzyme and the ionization of catalytic groups such as carboxyl or amino groups that are part of the enzyme's active site. The stability of the tertiary or quarternary structure of enzymes is also pH dependent and affects the velocity of the enzyme reaction, especially at extreme acidic or alkaline pHs. The pH for maximum stability of an enzyme does not necessarily coincide with the pH for maximum activity of that same enzyme. For example, the proteolytic enzymes trypsin and chymotrypsin are stable at pH 3, while they have maximum activity at pH 7–8.

To establish the pH optimum for an enzyme reaction, the reaction mixture is buffered at different pHs and the activity of the enzyme is determined. To determine pH enzyme stability relationships, aliquots of the enzyme are buffered at different pH values and held for a specified period of time (e.g., 1 hr). The pH of the aliquots is then adjusted to the pH optimum and each aliquot is assayed. The effect of pH on enzyme stability is thus obtained. These studies are helpful in establishing conditions for handling the enzyme and also may be useful in establishing methods for controlling enzyme activity in a food system. Note that pH stabil-

ity and the pH optimum for the enzyme activity are not true constants. That is to say, these may vary with particular source of enzyme, the specific substrate used, the temperature of the experiment, or even the buffering species used in the experiment. In the use of enzymes for analysis, it is not necessary that the reaction be carried out at the pH optimum for activity, or even at a pH at which the enzyme is most stable, but it is critical to maintain a fixed pH during the reaction (i.e., use buffer) and to use the same pH in all studies to be compared.
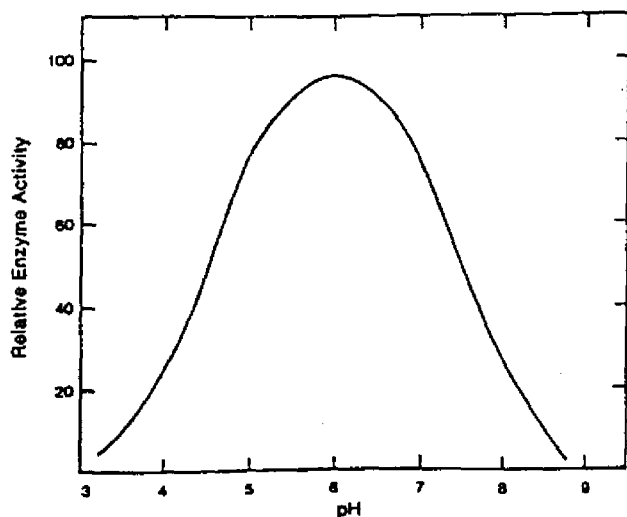
22.2.2.4 Activators and Inhibitors

22.2.2.4.1 Activators Some enzymes contain, in addition to a protein portion, small molecules that are activators of the enzyme. Some enzymes show an absolute requirement for a particular inorganic ion for activity while others show increased activity when small molecules are included in the reaction medium. These small molecules can play a role in maintaining the conformation of the protein, or they may form an essential component of the active site, or they may form part of the substrate of the enzyme.

In some cases, the activator forms a nearly irreversible association with the enzyme. These nonprotein portions of the enzyme are called prosthetic groups. The amount of enzyme activator complex formed is equal to the amount of activator present in the mixture. In these cases, activator concentration can be estimated up to concentrations equal to total enzyme concentration by simply measuring enzyme activity.

In most cases, dissociation constants for an enzyme activator complex are within the range of enzyme concentration. Dissociable nonprotein parts of enzymes are categorized as coenzymes. When this type of activator is added to enzyme, a curvilinear relationship similar to a Michaelis–Menten plot results, making difficult the determination of an unknown amount of activator. A reciprocal plot analogous to a Lineweaver–Burk plot can be constructed using standards and unknown activator concentrations estimated from such a plot.

An example of an essential activator is the pyridine coenzyme NAD⁺. NAD⁺ is essential for the oxidation of ethanol to acetaldehyde by alcohol dehydrogenase:

$$\text{ethanol} + \text{NAD}^+ \underset{\text{dehydrogenase}}{\overset{\text{alcohol}}{\rightleftharpoons}} \text{acetaldehyde} + \text{NADH} + \text{H}^+$$

[20]
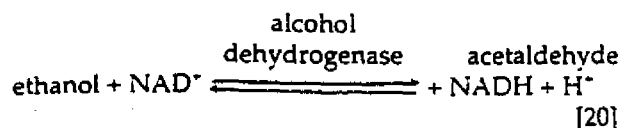
In the reaction, NAD⁺ is reduced to NADH and can be considered a second substrate. Another example of an activator of an enzyme is the chloride ion with α-amylase. In this case, α-amylase has some activity in the

Typical velocity–pH curve for an enzyme-catalyzed reaction. The maximum on the curve is the optimum for the system and can vary with temperature, specific substrate, and enzyme source.

absence of chloride. With saturating levels of chloride, the α-amylase activity increases about fourfold. Other anions, including F⁻, Br⁻, and I⁻, also activate α-amylase. These anions must not be in the reaction mixture if α-amylase stimulation is to be used as a method of determining chloride concentration.

22.2.2.4.2 Inhibitors
An enzyme inhibitor is a compound that when present in an enzyme-catalyzed reaction medium decreases the enzyme activity. Enzyme inhibitors can be categorized as irreversible or reversible inhibitors. Enzyme inhibitors include inorganic ions, such as Pb^{2+} or Hg^{2+}, which can react with sulfhydryl groups on enzymes to inactivate the enzyme, compounds that resemble substrate, and naturally occurring proteins that specifically bind to enzymes (such as protease inhibitors found in legumes).

1. Irreversible Inhibitors When the dissociation constant of the inhibitor enzyme complex is very small, the decrease in enzyme activity observed will be directly proportional to the inhibitor added. The speed at which the irreversible combination of enzyme and inhibitor reacts may be slow, and the effect of time on the reduction of enzyme activity by the addition of inhibitor must be determined to ensure complete enzyme–inhibitor reaction. For example, the amylase inhibitor found in many legumes must be preincubated under specified conditions with amylase prior to measurement of residual activity to accurately estimate inhibitor content (1).

2. Reversible Inhibitors Most inhibitors exhibit a dissociation constant such that both enzyme and inhibitor are found free in the reaction mixture. Several types of reversible inhibitors are known: competitive, noncompetitive, and uncompetitive.

Competitive inhibitors usually resemble the substrate structurally and compete with substrate for binding to the active site of the enzyme, and only one molecule of substrate or inhibitor can be bound to the enzyme at one time. An inhibitor can be characterized as competitive by adding a fixed amount of inhibitor to reactions at various substrate concentrations and by plotting the resulting data by the Lineweaver–Burk method and noting the effect of inhibitor relative to that of control reactions in which no inhibitor is added. If the inhibitor is competitive, the slope and x intercept of the plot with inhibitor are altered while the y intercept $(1/V_m)$ is unaltered. It can be shown that the ratio of the uninhibited initial velocity (v_0) to the inhibited initial velocity (v_i) gives:

$$\frac{v_0}{v_i} = \frac{[I]K_m}{K_i(K_m + [S])} \tag{21}$$

where:

K_i = dissociation constant of the enzyme–inhibitor complex

[I] = concentration of competitive inhibitor

Thus, a plot of v_0/v_i versus inhibitor concentration will give a straight line relationship. From this plot the concentration of a competitive inhibitor can be found (2).

A noncompetitive inhibitor binds to enzyme independent of substrate and is bound outside the active site of the enzyme. A noncompetitive inhibitor can be identified by its effect on the rate of enzyme-catalyzed reactions at various substrate concentrations and the data plotted by the Lineweaver–Burk method. A noncompetitive inhibitor will affect the slope and the y intercept as compared to the uninhibited system while the x intercept, $1/K_m$, is unaltered. Analogous to competitive inhibitors, a standard curve of v_0/v_i versus inhibitor concentration may be prepared and used to determine the concentration of a noncompetitive inhibitor (2).

Uncompetitive inhibitors bind only to the enzyme–substrate complex. Uncompetitive inhibition is noted by adding a fixed amount of inhibitor to reactions at several substrate concentrations and plotting the data by the Lineweaver–Burk method. An uncompetitive inhibitor will affect both the x and y intercepts of the Lineweaver–Burk plot as compared to the uninhibited system, while maintaining an equal slope to the uninhibited system (i.e., a parallel line will result). A plot of v_0/v_i versus inhibitor concentration can be prepared to be used as a standard curve for the determination of the concentration of an uncompetitive inhibitor (2).

22.2.3 Methods of Measurement

22.2.3.1 Overview

For practical enzyme analysis, it is necessary to be familiar with the methods of measurement of the reaction. Any physical or chemical property of the system that relates to substrate or product concentration can be used to follow an enzyme reaction. A wide variety of methods are available to follow enzyme reactions, including absorbance spectrometry, fluorimetry, manometric methods, titration, isotope measurement, and viscosity. A good example of the use of spectrophotometry as a method for following enzyme reactions is use of the spectra of the pyridine coenzyme NAD(H) and NADP(H), in which there is a marked change in absorbance at 340 nm upon oxidation–reduction (Fig. 22-10). Many methods depend on the increase or decrease in absorbance at 340 nm when these coenzymes are products or substrates in a coupled reaction.

An example of using several methods to measure
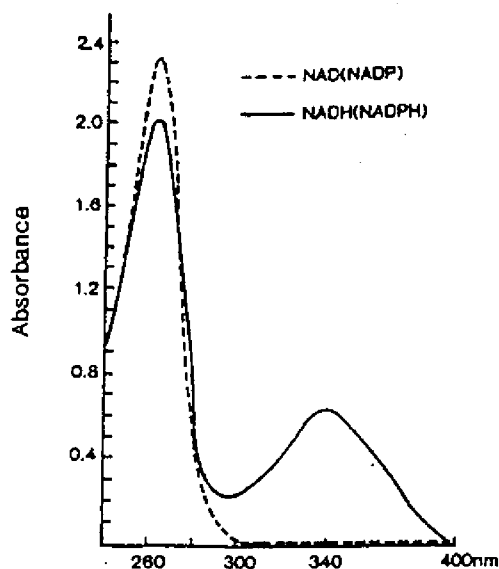
22-10 figure Absorption curves of NAD(P) and NAD(P)H; λ = wavelength. Many enzymatic analysis methods are based on the measurement of an increase or decrease in absorbance at 340 nm due to NAD(H) or NADP(H).

the activity of an enzyme is in the assay of α-amylase activity (3). α-Amylase cleaves starch at α-1,4 linkages in starch and is an endoenzyme. An endoenzyme cleaves a polymer substrate at internal linkages. This reaction can be followed by a number of methods, including reduction in viscosity, increase in reducing groups upon hydrolysis, reduction in color of the starch iodine complex, and polarimetry. However, it is difficult to differentiate the activity of α-amylase from β-amylase using a single assay. β-Amylase cleaves maltose from the nonreducing end of starch. While a marked decrease in viscosity of starch or reduction in iodine color would be expected to occur due to α-amylase activity, β-amylase can also cause changes in viscosity and iodine color if in high concentration. To establish whether α-amylase or β-amylase is being measured, the analyst must determine the change in number of reducing groups as a basis of comparison. Because α-amylase is an endoenzyme, hydrolysis of a few bonds near the center of the polymeric substrate will cause a marked decrease in viscosity, while hydrolysis of an equal number of bonds by the exo enzyme, β-amylase, will have little effect on viscosity.

In developing an enzyme assay, it is wise to first write out a complete, balanced equation for the particular enzyme-catalyzed reaction. Inspection of the products and substrates for chemical and physical properties that are readily measurable with available equipment will often result in an obvious choice of method for following the reaction in the laboratory.

If one has options in methodology, one should

select the method that is able to monitor the reaction continuously, is most sensitive, and is specific for the enzyme-catalyzed reaction.

22.2.3.2 Coupled Reactions

Enzymes can be used in assays via coupled reactions. Coupled reactions involve using two or more enzyme reactions so that a substrate or product concentration can be readily followed. In using a coupled reaction, there is an indicator reaction and a measuring reaction. For example:

$$S1 \xrightarrow{E1} P1 \qquad [22]$$

measuring reaction

$$P1 \xrightarrow{E2} P2 \qquad [23]$$

indicating reaction

The role of the indicating enzyme (E2) is to produce P2, which is readily measurable and, hence, is an indication of the amount of P1 produced by E1. Alternatively the same sequence can be used in measuring S1, the substrate for E1. When a coupled reaction is used to measure the activity of an enzyme (e.g., E1 above), it is critical that the indicating enzyme E2 not be rate limiting in the reaction sequence: the measuring reaction must always be rate determining. Consequently, E2 activity should be much greater than E1 activity for an effective assay. Coupled enzyme reactions can have problems with respect to pH of the system if the pH optima of the coupled enzymes are quite different. It may be necessary to allow the first reaction (e.g., the measuring reaction catalyzed by E1 above, Equation [22]) to proceed for a time and then arrest the reaction by heating to denature E1. The pH is adjusted, the indicating enzyme (E2, Equation [23]) added, and the reaction completed. If an endpoint method is used with a coupled system, the requirements for pH compatibility are not as stringent as for a rate assay because an extended time period can be used to allow the reaction sequence to go to completion.

22.3 APPLICATIONS

As described previously, certain information is needed prior to using enzyme assays analytically. In general, knowledge of K_m, time course of the reaction, the enzyme's specificity for substrate, the pH optimum and pH stability of the enzyme, and effects of temperature on the reaction and stability of the enzyme are desirable. Many times this information is available from the literature. However, a few preliminary experiments may be necessary, especially in the case of

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experiments in which velocities are measured. A time course to establish linearity of product formation or substrate consumption in the reaction is a necessity. An experiment to show linearity of velocity of the enzyme reaction to enzyme concentration is recommended (see Fig. 22-5).

22.3.1 Substrate Assays

The following is not an extensive compendium of methods for the measurement of food components by enzymatic analysis. Instead, it is meant to be representative of the types of analyses possible. The reader can consult handbooks published by the manufacturers of enzyme kits, for example, Boehringer–Mannheim (4), the review article by Whitaker (2), and the series by Bergmeyer (5) for a more comprehensive guide to enzyme methods applicable to foods.

22.3.1.1 Sample Preparation

Because of the specificity of enzymes, sample preparation prior to enzyme analysis is often minimal and may involve only extraction and removal of solids by filtration or centrifugation. Regardless, due to the wide variety of foods that might be encountered by the analyst using enzyme assays, a check should be made of the extraction and enzyme reaction steps by standard addition of known amounts of analyte to the food and extract, and measuring recovery of that standard. If the standard additions are fully recovered, this is a positive indication that the extraction is complete, that sample does not contain interfering substances that require removal prior to the enzymatic analysis, and that the reagents are good. In some cases, interfering substances are present but can be readily removed by precipitation or adsorption. For example, polyvinylpolypyrrolidone (PVPP) powder can be used to decolorize juices or red wines. With the advent of small syringe minicolumns (e.g., C18, silica, and ion-exchange cartridges), it is also relatively easy and fast to attain group separations to remove interfering substances from a sample extract.

22.3.1.2 Total Change/Endpoint Methods

While substrate concentrations can be determined in rate assays when the reaction is first order with respect to substrate concentration ($[S] << K_m$), substrate concentration can also be determined by the total change or endpoint method. In this method, the enzyme-catalyzed reaction is allowed to go to completion so that concentration of product, which is measured, is directly related to substrate. An example of such a system is the measurement of glucose using glucose oxidase and peroxidase, described below.

In some cases, an equilibrium is established in an endpoint method in which there is a significant amount of substrate remaining in equilibrium with product. In these cases, the equilibrium can be altered. For example, in cases in which a proton-yielding reaction is used, alkaline conditions (increase in pH) can be used. Trapping agents can also be used, in which product is effectively removed from the reaction, and by mass action the reaction goes to completion. Examples include the trapping of ketones and aldehydes by hydrazine. In this way, the product is continually removed and the reaction is pulled to completion. The equilibrium can also be displaced by increasing cofactor or coenzyme concentration.

Another means of driving a reaction to completion is a regenerating system (5). For example, in the measurement of glutamate, with the aid of glutamate dehydrogenase, the following can be done:

$$\begin{array}{ccc} & \text{glutamate} & \\ \text{glutamate} & \text{dehydrogenase} & \alpha\text{-ketoglutarate} \\ +\,NAD^+ & \rightleftharpoons & +\,NADH \qquad [24] \\ +\,H_2O & & +\,NH_4^+ \end{array}$$

$$\begin{array}{ccc} & \text{lactate} & \\ & \text{dehydrogenase} & \\ \text{pyruvate} & \longrightarrow & NAD^+ \qquad [25] \\ +\,NADH & & +\,\text{lactate} \\ +\,H^+ & & \end{array}$$
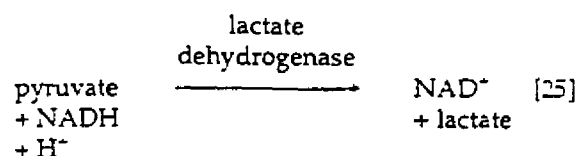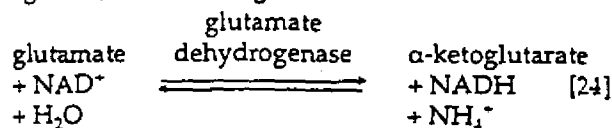
In this system, NADH is recycled to NAD^+ via lactate dehydrogenase until all the glutamate to be measured is consumed. The reaction is stopped by heating to denature the enzymes present, a second aliquot of glutamate dehydrogenase and NADH is added, and the α-ketoglutarate (equivalent to the original glutamate) measured via decrease in absorbance at 340 nm. An example in which the same equilibrium is displaced in the measurement of glutamate is as follows:

$$\begin{array}{ccc} & \text{glutamate} & \\ \text{glutamate} & \text{dehydrogenase} & \alpha\text{-ketoglutarate} \\ +\,NAD^+ & \rightleftharpoons & +\,NADH \qquad [26] \\ +\,H_2O & & +\,NH_4^+ \end{array}$$

$$\begin{array}{ccc} & \text{diaphorase} & \\ NADH +\,INT & \longrightarrow & NAD^+ \qquad [27] \\ & & +\,\text{formazan} \end{array}$$
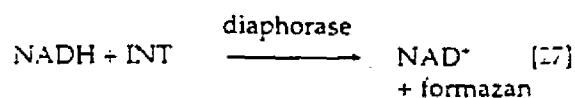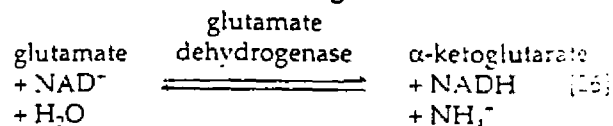
Iodonitrotetrazolium chloride (INT) is a trapping reagent for the NADH product of the glutamate dehydrogenase catalyzed reaction. The formazan formed is measurable colorimetrically at 492 nm.

22.3.1.3 Specific Applications

22.3.1.3.1 Measurement of Sulfite Sulfite is a food additive that can be measured by several techniques

including titration, distillation followed by titration, gas chromatography, and colorimetric analysis. Sulfite can also be specifically oxidized to sulfate by the commercially available enzyme sulfite oxidase (SO):

$$SO_3^{2-} + O_2 + H_2O \xrightarrow{\text{SO}} SO_4^{2-} + H_2O_2 \qquad [28]$$
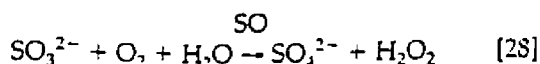
The H_2O_2 product can be measured by several methods including use of the enzyme NADH-peroxidase:

$$H_2O_2 + NADH + H^+ \xrightarrow{\text{NADH-peroxidase}} 2H_2O + NAD^+ \qquad [29]$$
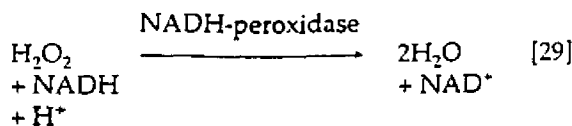
The amount of sulfite in the system is equal to the NADH oxidized, which is determined by decrease in absorbance at 340 nm. Ascorbic acid can interfere with the assay but can be removed by using ascorbic acid oxidase (6).

22.3.1.3.2 Colorimetric Determination of Glucose

The combination of the enzymes glucose oxidase and peroxidase can be used to specifically measure glucose in a food system (7). Glucose is preferentially oxidized by glucose oxidase to produce gluconolactone and hydrogen peroxide. The hydrogen peroxide plus o-dianisidine in the presence of peroxidase produces a yellow color that absorbs at 420 nm (Equations [30] and [31]). This assay is normally carried out as an endpoint assay and there is stoichiometry between the color formed and the amount of glucose in the extract, which is established with a standard curve. Because glucose oxidase is quite specific for glucose, it is a useful tool in determining the amount of glucose in the presence of other reducing sugars.

$$\beta\text{-D-glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \delta\text{-gluconolactone} + H_2O_2 \qquad [30]$$

$$H_2O_2 + o\text{-diansidine} \xrightarrow{\text{peroxidase}} H_2O + \text{oxidized dye (colored)} \qquad [31]$$
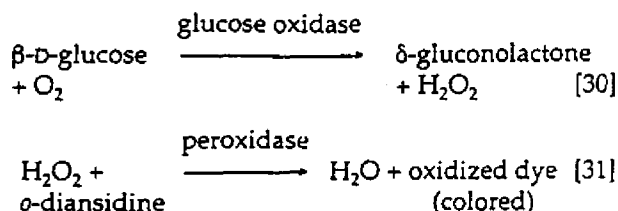
22.3.1.3.3. Starch/Dextrin Content
Starch and dextrins can be determined by enzymatic hydrolysis using amyloglucosidase, an enzyme that cleaves a-1,4 and a-1,6 bonds of starch, glycogen, and dextrins, liberating glucose (see Chapter 11). The glucose formed can be subsequently determined enzymatically. Glucose can be determined by the previously described colorimetric method, in which glucose is oxidized by glucose oxidase and coupled to a colored dye via reaction of the glucose oxidase product, hydrogen peroxide, with per-

oxidase. An alternative method of measuring glucose is by coupling hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6PDH) reactions:

$$\text{glucose} + ATP \xrightarrow{\text{HK}} \text{glucose-6-phosphate} + ADP \qquad [32]$$

$$\text{glucose-6-phosphate} + NADP^+ \xrightarrow{\text{G6PDH}} \text{6-phosphogluconate} + NADPH + H^+ \qquad [33]$$
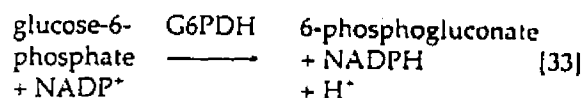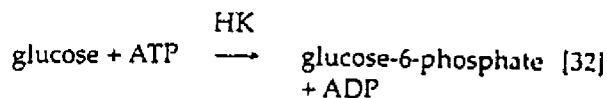
The amount of NADPH formed is measured by absorbance at 340 nm and is a stoichiometric measure of the glucose originating in the dextrin or starch hydrolyzed by amyloglucosidase. The amount of starch determined by this method is calculated as follows:

$$c = \frac{V\,MW}{\varepsilon\,b\,v\,1000} \times \Delta A_{340} \qquad [34]$$

where:

c = starch in sample solution (g/liter)
V = volume (ml) of reaction mixture
MW = molecular weight of starch
(Because this method measures glucose derived from starch, use 162.1, $MW_{glucose} - MW_{water}$.)
ε = absorption coefficient of NADPH at 340 nm (6.3 liter mmol^{-1} cm^{-1})
b = light pathlength of cuvette (1 cm)
v = volume of sample (ml)
$\Delta A_{340} = A_{340,sample} - A_{340,\,reagent\,blank}$

Note that hexokinase catalyzes the phosphorylation of fructose as well as glucose. The determination of glucose is specific because of the specificity of the second reaction, catalyzed by glucose-6-phosphate dehydrogenase, in which glucose-6-phosphate is the substrate.

This assay sequence can be used to detect the dextrins of corn syrup used to sweeten a fruit juice product. A second assay would be needed, however, without treatment with amyloglucosidase to account for the glucose in the product. The glucose determined in that assay would be subtracted from the result of the assay in which amyloglucosidase is used.

The same hexokinase glucose-6-phosphate dehydrogenase sequence used to measure glucose can also be used to measure other carbohydrates in foods. For example, lactose and sucrose can be determined via specific hydrolysis of these disaccharides by β-galactosidase and invertase respectively, followed by the use of the earlier described hexokinase, glucose-6-phosphate dehydrogenase sequence.

22.3.1.3.4 Determination of D-Malic Acid in Apple Juice

Two stereoisomeric forms of malic acid exist. L-Malic acid occurs naturally, while the D form is normally not found in nature. Synthetically produced malic acid is a mixture of these two isomers. Consequently, synthetic malic acid can be detected by a determination of D-malic acid. One means of detecting the malic acid is through the use of the enzyme decarboxylating D-malate dehydrogenase (8). Decarboxylating D-malate dehydrogenase (DMD) catalyzes the conversion of D-malic acid as follows:

$$\begin{array}{c} \text{DMD} \\ \text{D-malic acid} \xrightarrow{\hspace{1cm}} \text{pyruvate} + CO_2 \quad [35] \\ + NAD^+ \hspace{2cm} + NADH + H^+ \end{array}$$

The reaction can be followed by the measurement of NADH photometrically. Because CO_2 is a product of this reaction and escapes, the equilibrium of the reaction lies to the right and the process is irreversible. This assay is of value because the addition of synthetic D/L malic acid can be used to illegally increase the acid content of apple juice and apple juice products.

22.3.2 Enzyme Activity Assays

22.3.2.1 Peroxidase Activity

Peroxidase is found in most plant materials and is reasonably stable to heat. A heat treatment that will destroy all peroxidase activity in a plant material is usually considered to be more than adequate to destroy other enzymes and most microbes present. In vegetable processing, therefore, the adequacy of the blanching process can be monitored by following the disappearance of peroxidase activity (9). Peroxidase catalyzes the oxidation of guaiacol (colorless) in the presence of hydrogen peroxide to form tetraguaiacol (yellow brown) and water (Equation [36]). Tetraguaiacol has an absorbance maximum around 450 nm. Increase in absorbance at 450 nm can be used to determine the activity of peroxidase in the reaction mixture.

$$H_2O_2 + \text{guaiacol} \xrightarrow{\text{peroxidase}} \begin{array}{l} \text{tetraguaiacol} \\ \text{(colored)} \quad [36] \\ + H_2O \end{array}$$

22.3.2.2 Lipoxygenase

Recently it has been pointed out that lipoxygenase may be a more appropriate enzyme to measure the adequacy of blanching of vegetables than peroxidase (10). Lipoxygenase refers to a group of enzymes that catalyzes the oxidation by molecular oxygen of fatty acids

containing a cis, cis, 1,4-pentadiene system producing conjugated hydroperoxide derivatives:

$$\begin{array}{c} (-CH=CH-CH_2-CH=CH-) + O_2 \\ \text{lipoxygenase} \\ \xrightarrow{\hspace{1cm}} (-C-CH=CH-CH=CH-) \\ \hspace{2cm} | \\ \hspace{2cm} O \quad \text{(conjugated)} \quad [37] \\ \hspace{2cm} | \\ \hspace{2cm} O \\ \hspace{2cm} | \\ \hspace{2cm} H \end{array}$$

A variety of methods can be used to measure lipoxygenase activity in plant extracts. The reaction can be followed by measuring loss of fatty acid substrate, oxygen uptake, occurrence of the conjugated diene at 234 nm, or the oxidation of a cosubstrate such as carotene (11). All these methods have been used, and each has its advantages. The oxygen electrode method is widely used and replaces the more cumbersome manometric method. The electrode method is rapid and sensitive and gives continuous recording. It is normally the method of choice for crude extracts, but secondary reactions involving oxidation must be corrected for or eliminated. Zhang et al. (12) have reported the adaptation of the O_2 electrode method to the assay of lipoxygenase in green bean homogenates without extraction. Due to the rapidity of the method (<3 min including the homogenization), on-line process control using lipoxygenase activity as a control parameter for optimization of blanching of green beans is a real possibility. The formation of conjugated diene fatty acids with a chromophore at 234 nm can be followed continuously. However, optically clear mixtures are necessary. Bleaching of carotenoids has also been used as a measure of lipoxygenase activity. However, the stoichiometry of this method is uncertain, and all lipoxygenases do not have equal carotenoid bleaching activity. Williams et al. (10) have developed a semiquantitative spot test assay for lipoxygenase in which I^- is oxidized to I_2 in the presence of the linoleic acid hydroperoxide product and the I_2 detected as an iodine-starch complex.

22.3.2.3 Alkaline Phosphatase Assay

Alkaline phosphatase is a relatively heat-stable enzyme found in raw milk. The thermal stability o alkaline phosphatase in milk is greater than the non spore-forming microbial pathogens present in milk. The phosphatase assay is applied to dairy product to determine whether pasteurization has been done properly and to detect the addition of raw milk to pasteurized milk. The common phosphatase test is based on the phosphatase-catalyzed hydrolysis of disodi

umphenyl phosphate liberating phenol (13). The phenol product is measured colorimetrically after reaction with CQC (2,6-dichloroquinonechloroimide) to form a blue indophenol. The indophenol is extracted into *n*-butanol and measured at 650 nm. This is an example of a physical separation of product to allow the ready measurement of an enzyme reaction. Recently, a fluorometric assay has been suggested and has been commercialized for measurement of alkaline phosphatase in which the rate of fluorophore production can be monitored directly without butanol extraction used to measure indophenol when phenylphosphate is used as substrate (14). The fluorometric assay was shown to give greater repeatability compared to the standard assay in which phenylphosphate is used as substrate and was capable of detecting 0.05% raw milk in a pasteurized milk sample.

22.3.2.4 α-Amylase Activity

Amylase activity in malt is a critical quality parameter. The amylase activity in malt is often referred to as diastatic power and refers to the production of reducing substances by the action of α- and β-amylases on starch. The measurement of diastatic power involves digestion of soluble starch with a malt infusion (extract) and following increase in reducing substances by measuring reduction of Fehling's solution or ferricyanide. Specifically measuring α-amylase activity (often referred to as dextrinizing activity) in malt is more complicated and is based on using a limit dextrin as substrate. Limit dextrin is prepared by action of β-amylase (free of α-amylase activity) on soluble starch. The β-amylase clips maltose units off the nonreducing end of the starch molecule until an α-1,6-branch point is encountered. The resulting product is a β-limit dextrin that serves as the substrate for the endo cleaving α-amylase. A malt infusion is added to the previously prepared limit dextrin substrate and aliquots removed periodically to a solution of dilute iodine. The α-amylase activity is measured by changed color of the starch iodine complex in the presence of excess β-amylase used to prepare the limit dextrin. The color is compared to a colored disc on a comparator. This is continued until the color is matched to a color on a comparator. The time to reach that color is dextrinizing time and is a measure of α-amylase activity, a shorter time representing a more active preparation.

Because α-amylase is an endoenzyme, when it acts on a starch paste the viscosity of the paste is dramatically reduced, greatly influencing flour quality. Consequently, α-amylase activity is of great importance in whole wheat. Wheat normally has small amounts of α-amylase activity, but when wetted in the field, preharvest sprouting (pregermination) can occur in wheat,

with a dramatic increase in α-amylase activity. Preharvest sprouting cannot be easily detected visually, so measurement of α-amylase activity can be used as a sensitive estimate of preharvest sprouting. The falling number method is a procedure in which ground wheat is heated with water to form a paste, and the time it takes for a plunger to fall through the paste is recorded (15). Accordingly, the time in seconds (the falling number) is inversely related to the α-amylase activity and the degree of preharvest sprouting. This method of measuring enzyme activity is a good example of using change in physical property of a substrate as a means of estimation of enzyme activity.

22.3.2.5 Rennet Activity

Rennet, an extract of bovine stomach, is used as a coagulating agent in cheese manufacture. Most rennet activity tests are based on noting the ability of a preparation to coagulate milk. For example, 12% nonfat dry milk is dispersed in a 10 m*M* calcium chloride solution and warmed to 35°C. An aliquot of the rennet preparation is added and the time of milk clotting observed visually. The activity of the preparation is calculated in relationship to a standard rennet. As opposed to coagulation ability, rennet preparations can also be evaluated for proteolytic activity by measuring the release of a dye from azocasein (casein to which a dye has been covalently attached). In this assay, the rennet preparation is incubated with 1% azocasein. After the reaction period, the reaction is stopped by addition of trichloroacetic acid. The trichloroacetic acid precipitates the protein that is not hydrolyzed. The small fragments of colored azocasein produced by the hydrolysis of the rennet are left in solution and absorbance read at 345 nm (16, 17). This assay is based on the increase in solubility of a substrate upon cleavage by an enzyme.

22.3.3 Biosensors/Immobilized Enzymes

The use of immobilized enzymes as analytical tools is currently receiving increased attention. An immobilized enzyme in concert with a sensing device is an example of a biosensor. A biosensor is a device comprised of a biological sensing element (e.g., enzyme, antibody, etc.) coupled to a suitable transducer (e.g., optical, electrochemical, etc.). Immobilized enzymes, because of their stability and ease of removal from the reaction, can be used repeatedly, thus eliminating a major cost in enzyme assays. The most widely used enzyme electrode is the glucose electrode in which glucose oxidase is combined with an oxygen electrode to determine glucose concentration (18). When the electrode is put into a glucose solution, the glucose diffuses

into the membrane where it is converted to glucono-
lactone by glucose oxidase with the uptake of oxygen.
The oxygen uptake is a measure of the glucose concen-
tration. Glucose can also be measured by the action of
glucose oxidase with the detection of hydrogen perox-
ide, in which the hydrogen peroxide is detected amper-
ometrically at a polarized electrode (19). A large num-
ber of enzyme electrodes (biosensors) have been
reported in the literature recently. For example, a glyc-
erol sensor, in which glycerol dehydrogenase was
immobilized, has been developed for the determina-
tion of glycerol in wine (20). NADH produced by the
enzyme was monitored with a platinum electrode.
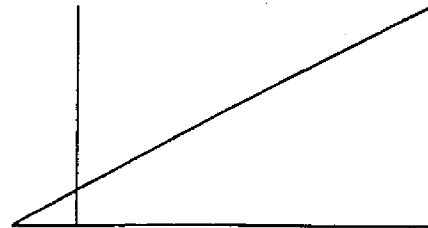
22.4 SUMMARY

Enzymes, due to their specificity and sensitivity, are
valuable analytical devices for quantitating com-
pounds that are enzyme substrates, activators, or
inhibitors. In enzyme-catalyzed reactions, the enzyme
and substrate are mixed under specific conditions (pH,
temperature, ionic strength, substrate concentration,
and enzyme concentrations). Changes in these condi-
tions can affect the reaction rate of the enzyme and
thereby the outcome of the assay. The enzymatic reac-
tion is followed by measuring either the amount of
product generated or the disappearance of the sub-
strate. Applications for enzyme analyses will increase
as a greater number of enzymes are purified and
become commercially available. In some cases, gene
amplification techniques will make enzymes available
that are not naturally found in great enough amounts to
be used analytically. The measurement of enzyme activ-
ity is useful in assessing food quality and as an indica-
tion of the adequacy of heat processes such as pasteur-
ization and blanching. In the future, as in-line process
control (to maximize efficiencies and drive quality
developments) in the food industry becomes more
important, immobilized enzyme sensors, along with
microprocessors, will likely play a prominent role.

22.5 STUDY QUESTIONS

1. The Michaelis–Menten equation mathematically defines
   the hyperbolic nature of a plot relating reaction velocity to
   substrate concentration for an enzyme-mediated reaction.
   The reciprocal of this equation gives the Lineweaver–Burk
   formula and a straight-line relationship as shown below.
   a. Define what v_0, K_m, V_m, and [S] refer to in the Line-
      weaver–Burk formula.
   b. Based on the components of the Lineweaver–Burk for-
      mula, label the y-axis, x-axis, slope, and y-intercept on
      the plot.
   c. What factors that control or influence the rate of
      enzyme reactions affect K_m and V_m?

$$\frac{1}{v_0} = \frac{K_m}{V_m}\frac{1}{[S]} + \frac{1}{V_m}$$

a. $v_0 =$
   $K_m =$
   $V_m =$
   [S] =
b.

c. $K_m -$
   $V_m -$

2. Explain, on a chemical basis, why extremes of pH and
   temperature can reduce the rate of enzyme-catalyzed
   reactions.
3. Differentiate among competitive, noncompetitive, and
   uncompetitive enzyme inhibitors.
4. You believe that the food product you are working with
   contains a specific enzyme inhibitor. Explain how you
   would quantitate the amount of enzyme inhibitor (I) pres-
   ent in an extract of the food. The inhibitor (I) in question
   can be purchased commercially in a purified form from a
   chemical company. The inhibitor is known to inhibit the
   specific enzyme E, which reacts with the substrate S to
   generate product P, which can be quantitated spectropho-
   tometrically.
5. What methods can be used to quantitate enzyme activity
   in enzyme-catalyzed reactions?
6. What is a coupled reaction, and what are the concerns in
   using coupled reactions to measure enzyme activity? Give
   a specific example of a coupled reaction used to measure
   enzyme activity.
7. Explain how D-malic acid can be quantitated by an enzy-
   matic method to test for adulteration of apple juice.
8. Why is the enzyme peroxidase often quantitated in pro-
   cessing vegetables?
9. Explain the purpose of testing for phosphatase activity in
   the dairy industry, and explain why it can be used in that
   way.
10. The falling number value often is one of the quality con-
    trol checks in processing cereal-based products. What is
    the falling number test, and what information does it pro-
    vide? What other tests could be used to assay this quality
    factor?
11. Explain how glucose can be quantitated using a specific
    immobilized enzyme.

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Analysis for Extraneous Matter

John R. Pedersen

23.1 INTRODUCTION

Analysis for extraneous matter is an important element both in the selection of raw materials for food manufacturing and for monitoring the quality of processed foods.

23.1.1 Food, Drug, and Cosmetic Act

The Federal Food, Drug, and Cosmetic Act (FD&C Act) of 1938 with Amendments (1) defines a food as adulterated "if it consists in whole or in part of any filthy, putrid, or decomposed substance, or if it is otherwise unfit for food [Section 402 (a)(3)]; or if it has been prepared, packed, or held under insanitary conditions whereby it may have become contaminated with filth, or whereby it may have been rendered injurious to health" [Section 402 (a)(4)]. The filthy, putrid, or decomposed substances referred to in the law include the extraneous matter addressed in this chapter. In addition, extraneous matter includes adulterants that may be encountered in processing systems, such as lubricants, metal particles, or other contaminants (animate or inanimate) that may be introduced into a food intentionally or because of a poorly operated food processing system.

23.1.2 Good Manufacturing Practices

The Current Good Manufacturing Practice in Manufacturing, Packing, or Holding Human Food (GMPs) was published by the Food and Drug Administration (FDA) (2) to provide guidance for compliance with the FD&C Act (see also Chapter 2). That regulation provides guidelines for operating a food processing facility in compliance with Section 402 (a)(4). Paramount to complying with the FD&C Act and GMPs is the thorough inspection of raw materials and routine monitoring of food processing operations to ensure protection of the consuming public from harmful or filthy food products.

23.1.3 Defect Action Levels

Most of our foods are made from or consist in part of ingredients that are obtained from plants or animals and are mechanically stored, handled, and transported in large quantities. It would be virtually impossible to keep those materials completely free of various forms of contaminants. In recognition of that, the FDA (3) has established defect action levels (DALs) that reflect current maximum levels for natural or unavoidable defects in food for human use that present no health hazard. They reflect the maximum levels that are considered unavoidable under good manufacturing practices, and

apply mainly to contaminants that are unavoidably carried over from raw agricultural commodities into the food processing system. The manner in which foods are manufactured may lead to their contamination with extraneous materials if strict controls in processing are not maintained. This latter kind of contamination is not excused by the DALs.

The most current information of FDA laws and regulations relevant to extraneous matter, including DALs, can be found on the Internet (FD&C Act - http://www.fda.gov/opacom/laws/fdcact/fdctoc.html DALs - http://vm.cfsan.fda.gov/~dms/dalbook.html)

23.1.4 Purposes of Analyses

The major purposes for conducting analyses for extraneous matter in foods are to ensure the protection of the consuming public from harmful or filthy food products, to meet regulatory requirements of the FD&C Act Sections 402 (a)(3) and 402 (a)(4), and to comply with defect action levels.

23.2 GENERAL CONSIDERATIONS

23.2.1 Methodologies

There are various methods for separating (isolating) extraneous materials from foods and for identifying and enumerating them. The most authoritative source, and that generally considered official by the FDA, is the Official Methods of Analysis of AOAC International. Chapter 16, Extraneous Materials: Isolation (4), includes methods for extraneous matter isolation in 16 food categories, including dairy products; grains and their products; poultry, meat, fish, and other marine products; snack food products; spices and other condiments; animal excretions; and mold and rot fragments.

The American Association of Cereal Chemists (AACC) (5) has established methods for isolating and identifying extraneous matter in cereal grains and their products (AACC Method 28-00). In most instances, the AACC methods are based on FDA or AOAC methods, but the format is slightly different. The AACC presents each procedure in an outline form that includes the scope, apparatus, and reagents required and the procedure in itemized steps; the AOAC uses a narrative paragraph form.

A valuable resource on analysis for extraneous matter is Principles of Food Analysis for Filth, Decomposition and Foreign Matter, FDA Technical Bulletin No. 1 (6). The FDA Training Manual for Analytical Entomology in the Food Industry (7) is a looseleaf publication prepared to facilitate the orientation of food analysts to the basic techniques they will need for filth analysis. A recent, more advanced resource is Fundamentals of

Microanalytical Entomology—A Practical Guide to Detecting and Identifying Filth in Foods (8). Most chapter authors of this resource are, or have been, FDA personnel "involved in the forensic aspect of piecing together the etiological puzzles of how insect filth gets into processed food products" (8). The authors share their experience gained in gathering and developing evidence used to document violations of the law that the FDA is mandated to enforce.

23.2.2 Definition of Terms

Terms used by AOAC International to classify or characterize various types of extraneous materials are defined as follows.

23.2.2.1 Extraneous Materials

Any foreign matter associated with objectionable conditions or practices in production, storage, or distribution; included are various classes of filth, decomposed material (decayed tissues due to parasitic or nonparasitic causes), and miscellaneous matter such as sand and soil, glass, rust, or other foreign substances. Bacterial counts are not included.

23.2.2.2 Filth

Any objectionable matter contributed by animal contamination such as rodent, insect, or bird matter; or any other objectionable matter contributed by unsanitary conditions.

23.2.2.3 Heavy Filth

Heavier material separated from products by sedimentation based on different densities of filth, food particles, and immersion liquids. Examples of such filth are sand, soil, and some animal excreta pellets.

23.2.2.4 Light Filth

Lighter filth particles that are oleophilic and are separated from products by floating them in an oil–aqueous liquid mixture. Examples are insect fragments, whole insects, rodent hairs and fragments, and feather barbules.

23.2.2.5 Sieved Filth

Filth separated from food products on the basis of particle size using selected sieve mesh sizes.

23.2.2.6 Diagnostic Characteristics of Filth

Examples include specific diagnostic characteristics of molds (i.e., parallel hyphal walls, septation, granular

appearance of cell contents, branching of hyphae, blunt ends of hyphal filaments, nonrefracted appearance of hyphae); diagnostic characteristics of insect fragments (i.e., recognizable shape, form, or surface sculpture, an articulation or joint, setae or setal pits, sutures), rodent hairs (i.e., pigment patterns and structural features), feather barbules (i.e., structural features); diagnostic characteristics of insect-damaged grains and packaging materials; and chemical identification of animal urine and excrement.

23.2.3 Objectivity/Subjectivity of Methods

Insect parts, rodent hairs, and feather barbules in food products are generally reported as the total number of filth elements counted of each kind encountered per sample unit. They are identified on the basis of objective criteria (see section 23.2.2.6 above). However, identifying insect fragments is not a simple task. Training and supervised practice are required to achieve competence and consistency. Some fragments are easily identified on the basis of structural shape and form. Mandibles, for example, are quite distinctive in their shape and configuration; certain species of insects can be determined on the basis of this one structure. In other instances, fragments may be mere chips of insect cuticle that have neither distinctive shape nor form but can be identified as being of insect origin if they have one or more of the characteristics given in section 23.2.2.6. Experienced analysts should rarely misinterpret fragments.

Isolation of extraneous material from a food product so that it can be identified and enumerated can be a very simple procedure or one that requires a series of several rather involved steps. In the process of isolating fragments from flour by the acid hydrolysis method, the sample is transferred from the digestion container to the separatory container and then to the filter paper for identification and enumeration. At each of these transfers there is an opportunity for loss of fragments. Although the analyst may have made every effort to maintain the isolation "quantitative," there are opportunities for error. Both fragment loss and analysis variation are minimized by common use of standard methods and procedures and proper training and supervised practice.

Another concern involves the significance of insect fragment counts (as well as particles of sand, pieces of rodent excreta, rodent hairs, etc.) in relation to fragment size. Fragment counts are reported on a numerical basis; they do not reflect the total contaminant biomass that is present. A small fragment is counted the same as a large fragment. The size of the fragment may be a reflection of the process to which a common raw material, such as wheat, has been subjected, a more vigorous process producing more and smaller frag-

ments than a less vigorous process. These factors have been of concern to food processors for some time and have prompted the search for more objective means of determining insect contamination.

23.3 METHODS

Various basic methods for isolation of extraneous matter were suggested in section 23.2.2, which defined different types of filth: separation on the basis of differences in density, affinity for oleophilic solvents, particle size; diagnostic characteristics for identification of filth; and chemical identification of contaminants. Not all methods of analysis for extraneous matter for all categories of food can be discussed in this chapter. However, a few representative methods have been selected to illustrate the various principles of separation and isolation. As indicated, some isolations are effected very simply and others are more complex, requiring a series of various steps. AOAC International methods (4) are used as reference sources, but they will be presented in a manner similar to that used for AACC methods (5). Refer to the specific AOAC methods cited for detailed instructions of the procedures.

23.3.1 Foreign Matter in Spices and Condiments—Sieving Method (AOAC Method 960.51)

23.3.1.1 Scope

Applicable to ground allspice, anise, curry powder, dill seed, fennel, fenugreek, poppyseed, savory, and condiments; heavy filth only: caraway seed, cardamon, celery seed, cloves, coriander, cumin, ginger, mace, marjoram, mustard, oregano, rosemary, sage, and thyme.

23.3.1.2 Apparatus

1. No. 20 sieve (850-μm openings) and pan
2. Widefield stereoscopic microscope

23.3.1.3 Reagents

There are no reagents in this procedure.

23.3.1.4 Procedure

1. Sift 200–400 g of ground spice through No. 20 sieve.
2. Transfer insects or other filth retained on sieve to suitable dish and examine with widefield stereoscopic microscope.

23.3.1.5 Explanation

This method illustrates a very simple technique based on particle size separation. The No. 20 sieve refers to the National Institute of Standards and Technology (formerly called the United States National Bureau of Standards) sieve with 20 mesh per inch (plain weave), which provides a sieve opening of 850 μm. That sieve is coarse enough to let the ground spices pass through and yet retain the adult stages of insects and other contaminants of species and condiments. The analyst must then be able to identify the insect species, stage, and whether live or dead, as well as any other foreign objects.

23.3.2 Filth in Shelled Nuts—Heavy Filth By Sedimentation (AOAC Method 968.33a)

23.3.2.1 Scope

Applicable to shelled nuts except pecans.

23.3.2.2 Apparatus

1. 600-ml beaker
2. Ashless filter paper
3. Stereoscopic microscope

23.3.2.3 Reagents

Note: Use effective fume removal device to remove vapors of flammable solvents (petroleum ether) and toxic solvents (carbon tetrachloride and chloroform).

1. Petroleum ether (pet ether)
2. Chloroform ($CHCl_3$)
3. Carbon tetrachloride (CCl_4)

23.3.2.4 Procedure

1. Weigh 100-g sample into a 600-ml beaker.
2. Add ca. 350 ml pet ether and boil gently 30 min, adding pet ether to maintain original volume. (Note: petroleum ether is extremely flammable.)
3. Decant solvent, taking care not to lose any coarse nut tissue, and discard.
4. Add ca. 300 ml of $CHCl_3$ to beaker and let settle 10–15 min.
5. Pour off floating nut meats and about two thirds of the $CHCl_3$ and discard.
6. Repeat separation with smaller volumes of mixture of $CHCl_3$ and CCl_4 (1 + 1) until residue in beaker is relatively free of nutmeat particles.
7. Transfer residue in beaker to ashless paper and examine for heavy filth.

8. Count number of particles of shell, sand, and soil.

9. If appreciable amount of sand or soil is present, ignite paper in weighed crucible at ca. 500°C and weigh. Report residue as percent of 100-g sample.

23.3.2.5 Explanation

The petroleum ether is used as a means for defatting the nut meats in preparation for continued analysis to determine light filth by an additional procedure. The chloroform and chloroform–carbon tetrachloride solvents allow pieces of shell, sand, and soil to settle the bottom of the beaker on the basis of specific gravity and cause the defatted nut meats to float and be decanted. Although the AOAC method does not specify microscopic examination, that is desirable to identify the extraneous material.

Essentially the same procedure is used to isolate pieces of rodent excreta from corn grits, rye and wheat meal, whole wheat flour, farina, and semolina in AOAC Method 941.16A. It should be noted that the use of the more toxic solvents such as carbon tetrachloride, chloroform, and petroleum ether is avoided in most contemporary analytical methods.

23.3.3 Insect Infestation (Internal) of Wheat—Cracking Flotation Method (AOAC Method 982.31)

23.3.3.1 Scope

Applicable to wheat. [Also available are specific cracking flotation methods for oats (AOAC Method 985.36) and grains and seeds (except wheat and oats) (AOAC Method 955.42).]

23.3.3.2 Apparatus

1. Jones sampler (riffle-type divider)
2. No. 12 sieve (1680-μm openings), No. 100 sieve (150-μm openings)
3. Cutting-type mill
4. 2-liter glass beaker
5. Magnetic stirring hotplate
6. 2-liter trap flask (Wildman)
7. Wide-stem funnel
8. Filter flask
9. Ruled filter paper—smooth, high wet strength, rapid acting filter paper ruled with oil-, alcohol-, and waterproof lines 5 mm apart
10. Widefield stereoscopic microscope, 15×

23.3.3.3 Reagents

1. Hydrochloric acid (HCl)
2. 40% isopropanol

3. Tween 80:40% isopropanol mixture—to 40 ml of polysorbate 80 (ICI Americas, Inc.) add 210 ml 40% isopropanol, mix, and filter.
4. Na₄EDTA:40% isopropanol mixture—dissolve 5 g of Na₄EDTA in 100 ml of H₂O, add 100 ml of isopropanol, mix, and filter.
5. Mineral oil—paraffin oil, white, light, 125/135 Saybolt Universal Viscosity (38°), sp. gr. 0.840–0.860 (24°C)

23.3.3.4 Procedure

1. Sample preparation
   a. Mix grain by passing six times through Jones divider (Fig. 23-1), recombining before each pass.
   b. Separate out slightly more than 50 g and weigh 50 g.
   c. Transfer weighed sample to No. 12 sieve (Fig. 23-2) and work any visible insects thru sieve using stiff brush.
   d. Grind sieved sample in cutting-type mill set at 0.061 in.
2. Isolation
   a. Transfer cracked wheat to 2-liter glass beaker containing magnetic stirring bar and mixture of 600 ml of H₂O + 50 ml of HCl. Stir *gently* while boiling 15 min on hotplate.
   b. Transfer sample to No. 100 sieve with a gen-
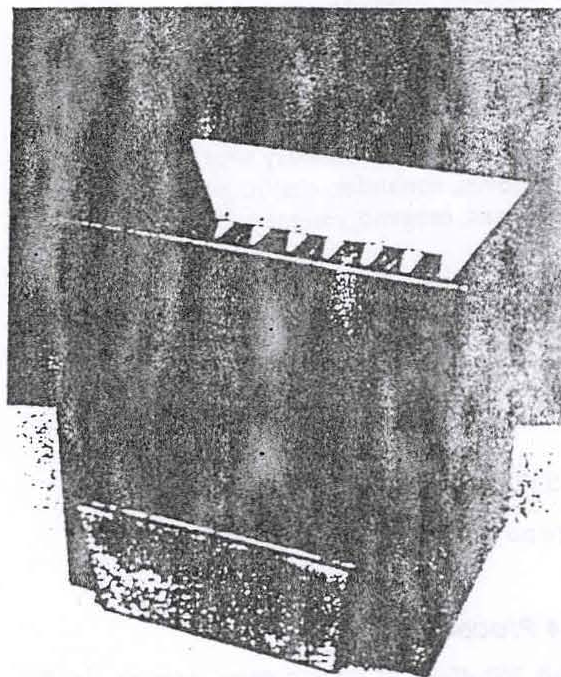
23-1 figure Jones sampler (also called a Jones divider; riffle divider). Alternate channels cause grain and other flowable solids to be systematically divided into representative fractions.
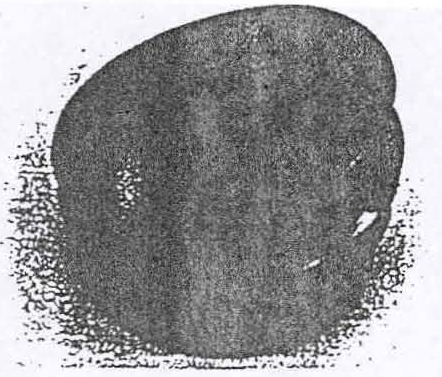
23-2 figure Sieve and pan. Standard mesh sizes are available for various particle size separations.

tle stream of hot tap water. Wash material on sieve with *very gentle* stream of hot (55–70°C) tap water until washings show no acidity when tested with blue litmus paper.

c. Place a widestem funnel in the neck of a 2-liter Wildman trap flask (Fig. 23-3) containing a magnetic stirring bar. Quantitatively transfer the residue from the sieve to the flask with 40% isopropanol. Add 40% isopropanol to make 800 ml total volume.

d. Clamp stirring rod of trap flask so stopper is above liquid in flask. Stir gently while boiling 7 min on magnetic stirring hotplate.

e. Remove flask from hotplate and wash down sides with minimal 40% isopropanol.

f. Immediately add 100 ml 1 + 1 mixture of Tween 80:40% isopropanol solution and Na_4EDTA:40% isopropanol solution slowly down stirring rod. Hand stir *gently* 1 min and let stand 3 min.

g. Add 50 ml of mineral oil by pouring down stirring rod. Stir magnetically 5 min on cool stirrer and let stand 3 min.

h. Fill flask with 40% isopropanol, added slowly down stirring rod to avoid agitation of flask contents, and let stand 20 min *undisturbed*.

i. Trap off oil layer, rinsing neck of flask with 40% isopropanol. Add rinse to trappings in beaker.

j. Add 35 ml of mineral oil to flask and hand stir 1 min. Clamp stirring rod with stopper at midpoint of flask and let stand for 5 min; then spin stirring rod to free settlings from top of stopper and adjust oil level to ca. 1 cm above fully raised stopper. Let stand *undisturbed* 15 min.

k. Trap off oil layer, combining trappings with those in beaker. Rinse neck of flask well with 40% isopropanol and add rinsings to beaker.
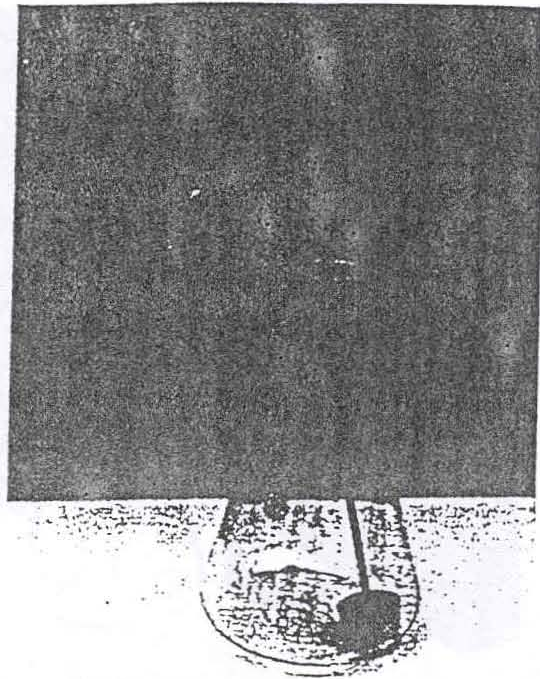
23-3 figure Wildman trap flask. Stopper on shaft is lifted up to neck of flask to trap off floating layer.

l. Filter trappings/rinsings on ruled filter paper, rinsing beaker well with isopropanol (Fig. 23-4).

m. Examine filter paper at 15x, counting only whole or equivalent insects and cast skins (Fig. 23-5).

23.3.3.5 Explanation

This method was developed to replace a previous simpler method that resulted in excessive amounts of plant debris trapped on filter papers. The previous method required as many as 5–10 filter papers to collect material trapped from a 100-g sample of wheat.

The initial screening with the No. 12 sieve (Fig. 23-2) is to remove any forms of insect that may be outside the sample wheat kernels.

A 50-g sieved wheat sample is ground to a particle size roughly that of coarsely ground coffee to expose and release insect forms that exist inside the wheat kernels. Although there is no official action level for internal infestation in wheat, the insects that develop inside wheat kernels are the primary source of insect fragments in flour, for which there is a DAL of 75 fragments (average) per 50 g in six subsamples (9).

The sample is boiled in hydrochloric acid solution to hydrolyze the starch so that it does not interfere with the flotation separation of insect contaminants. The hydrolyzed starch is washed from the sample with hot
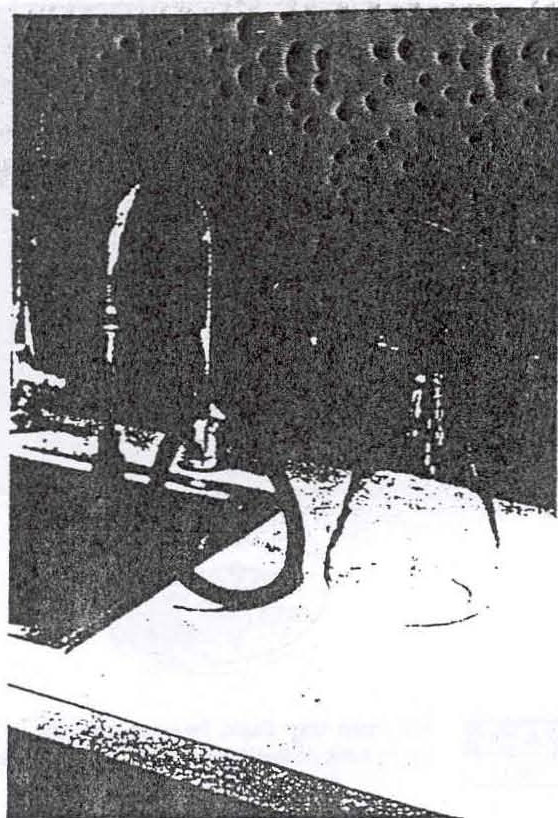
23-4 figure Filter flask and funnel. A special designed funnel has a collar (partially raised) that holds ruled filter paper in place on the funnel base for trapping filth for examination. Suction is applied with a water aspirator.

23-5 figure Widefield stereoscopic microscope. Used to examine ruled filter papers for insect fragments, rodent hairs, feather barbules, and other microscopic contaminants.

tap water while the sample and contaminants are retained on the No. 100 sieve. The tap water also removes the acid to prevent any chemical reaction with Na_4EDTA used later in the procedure.

After the sample has been washed and neutralized, it is transferred to a Wildman trap flask (Fig. 23-3) to separate any insect material present from the remaining plant material. The sample is boiled in 40% isopropanol to deaerate the remaining plant material to prevent particles from being trapped off with the contaminants. The mixture of Tween 80 and Na_4EDTA solutions serves to prevent lighter plant materials, such as particles of bran, from being trapped off with the contaminants.

Tween 80 (polyoxyethylene sorbitan monooleate) is a nonionic agent used in many microscopic filth procedures. It appears to have certain surface active properties that make it a useful adjunct to Na_4EDTA. Na_4EDTA in the presence of Tween 80 appears to be a depressor for food materials (such as bran and other light plant matter) that otherwise tend to float. It has been suggested that the chelating properties of Na_4EDTA may result in its adsorption onto the surfaces of food particles along with the surfactant Tween 80, thereby preventing an attraction of the food particles to

oils used to separate out insect contaminants. By preventing plant material from being collected in the mineral oil layer that is trapped off, contaminants such as oleophilic insect parts (exoskeleton) that are contained in the separating oil are much easier to distinguish and identify.

23.3.4 Light Filth (Pre- and Post-Milling) in Flour (White)—Flotation Method (AOAC Method 972.32)

23.3.4.1 Scope

Applicable to wheat flour (white).

23.3.4.2 Apparatus

1. 2–2.5-liter beaker and 1-liter beaker
2. Autoclave
3. Magnetic stirrer hotplate
4. Kilborn funnel or percolator
5. Ruled filter paper

23.3.4.3 Reagents

1. HCl solution
2. Mineral oil-paraffin, light 125/135 Saybolt

3. 5% detergent solution (aqueous Na lauryl sulfate)

23.3.4.4 Procedure

1. Digest (hydrolyze) 50 g of flour in 2–2.5-liter beaker with 600 ml of HCl solution (3 + 97) in autoclave 5 min at 121°C.
2. Immediately transfer digest to 1-liter beaker using HCl (3 + 97) at room temperature to assist transfer.
3. Add 50 ml of mineral oil and stir magnetically 5 min.
4. Quantitatively transfer sample to Kilborn funnel or percolator (Fig. 23-6). Retain beaker.
5. Let stand 30 min, stirring gently with long glass rod several times during first 10 min.
6. Drain lower layer to ca. 3 cm of interface, wash sides of funnel with cold tap water, and let layers separate ca. 2–3 min. Repeat drain and H_2O wash until lower phase is clear.
7. After final wash, drain oil layer into retained beaker, rinsing sides of funnel with H_2O and alcohol.
8. Add HCl to ca. 3% (vol/vol) and boil 3–4 min on hot plate.
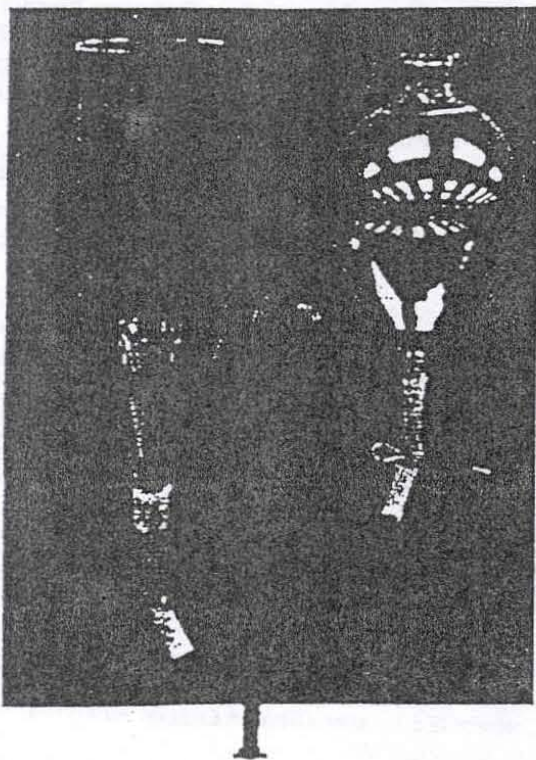
9. Filter hot solution through ruled filter paper (Fig. 23-4) and thoroughly rinse beaker and funnel with H_2O, alcohol, and 5% detergent solution onto ruled filter paper.
10. Examine microscopically (Fig. 23-5) and record light filth (insect fragments, rodent hairs and fragments, and feather barbules).

23.3.4.5 Explanation

This is a common method used for insect fragments, rodent hairs, and other forms of light filth determination. The acid digestion breaks down the starch in the flour and allows the other flour constituents to more cleanly separate from the dilute acid solution. Although the AOAC method calls for digestion by autoclaving, AACC Method 28-41A provides for an alternative hotplate digestion, which might be more convenient for some laboratories. The oleophilic property of insect fragments, rodent hairs, and feather barbules allows them to be coated by the mineral oil and trapped in the oil layer for separation and collection on ruled filter paper. The heavier sediments of the digestion are washed and drained from the funnel. Fragments and rodent hairs are reported on the basis of 50 g of flour. The current FDA DAL for insect fragments in flour is an average of 75 or more per 50 g in six subsamples, and an average of one or more rodent hairs per 50 g in six subsamples (9).

23.4 COMPARISON OF METHODS

There are limited sources of methods for isolation of extraneous materials from foods. AOAC International methods (4) are generally considered official and are often the basis for methods recommended by other organizations, such as the AACC (5). The difference in format used by the AACC has already been mentioned. The AACC has included colored pictures of representative insect fragments commonly found in cereal products, pictorial examples of rodent hair structure, and radiographic examples of grain kernels that contain internal insect infestation. Kurtz and Harris (10) provide a virtual parts catalog of insect fragments with a series of micrographs. Gentry et al. (11) is an updated version of the Kurtz and Harris publication and includes colored micrographs of common insect fragments.

23.5 OTHER TECHNIQUES

Although X-ray radiography is not an official method for isolation of extraneous material, it is used by some processors as a means of inspecting wheat for internal insect infestation, the source of insect fragments in

23-6 figure Separatory funnel(s). Straight-sided, open-top funnel (left); leveling-bulb type with narrow opening at top (right). These types may be used to substitute for the Kilborn funnel or percolator.

processed cereal products (12). The availability of suitable equipment may be a limiting factor in the use of X-ray radiography.

Mention was made earlier of the subjectivity involved in the isolation and especially identification of some biological contaminants commonly assessed in food products. Results have been based on numbers of particular contaminants rather than on mass determined in an objective manner. Some research has been directed at measuring uric acid content of samples as a basis for measuring insect or bird contamination by excrement (13). AOAC International has three methods for uric acid detection (Methods 962.20, 986.29, 969.46).

More recently, an ELISA (enzyme-linked immunosorbent assay) method (see Chapter 21) has been developed to objectively measure quantitatively the amount of insect material in a sample (14). It involves the measurement of the insect protein myosin. The advantage of uric acid and the myosin ELISA techniques is their adaptability to automation and objective measurement. Until these methods are accepted, one of the major needs for satisfactory filth isolation is properly trained laboratory analysts.

Methods described above in section 23.3 are directed primarily at routine quality control efforts to determine if the level of natural or unavoidable defects is below the defect action level. To a certain extent, those routine methods can be used to identify the source of contaminants in processed foods. However, other more sophisticated techniques offer opportunities to pinpoint the nature and source of contaminants that may exist unavoidably or due to mistakes, accidents, material or equipment failure, or intentional adulteration.

Microscopy techniques including light microscopy, fluorescence microscopy, and scanning electron microscopy (SEM) are used to study the structure/function relationships of food (15), but also can be applied to questions of extraneous matter. For example, SEM with energy dispersive spectroscopy (EDS) can be used to determine the nature of metals in products that may be due to equipment failure or intentional adulteration due to tampering (16). Light microscopy in a polarized mode can be used to distinguish between plastics, glass, and other fiber or crystalline contaminants (17).

23.6 SUMMARY

Extraneous matter in raw ingredients and in processed foods might be unavoidable in the array of foods that are stored, handled, processed, and transported. Defect action levels may be set for amounts considered unavoidable and no health hazard. A variety of methods are available to isolate extraneous matter from

foods. Those methods largely prescribed by AOAC International employ a series of physical and chemical means to separate the extraneous material for identification and enumeration. Major concerns in the analysis of food products for extraneous matter are the objectivity of methods and the availability of adequately trained analysts.

23.7 STUDY QUESTIONS

1. Indicate why the FDA has established defect action levels.
2. List three major reasons for conducting analysis for extraneous matter in foods.
3. What two resources provide methods for separating extraneous matter from cereal grains and their products?
4. There are several basic principles involved in separating (isolating) extraneous matter from foods. List five of these principles and give an example of each principle.
5. Briefly describe the major constraint(s) to currently accepted methods for analyses of extraneous matter in foods.
6. Explain how some of the more recent analytical techniques can assist in identifying sources of extraneous matter in foods.

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24

chapter

Determination of Oxygen Demand

Yong D. Hang

24.1 INTRODUCTION

Oxygen demand is a commonly used parameter to evaluate the potential effect of organic pollutants on either a wastewater treatment process or a receiving water body. Because microorganisms utilize these organic materials, the concentration of dissolved oxygen is greatly depleted from the water. The oxygen depletion in the environment can have a detrimental effect on fish and plant life.

The three main methods used to measure the oxygen demand of water and wastewater are biochemical oxygen demand (BOD), chemical oxygen demand (COD), and total organic carbon (TOC). This chapter briefly describes the principles, procedures, applications, and limitations of each method. Methods described are from *Standard Methods for the Examination of Water and Wastewater*, published by the American Public Health Association (APHA). Refer to methods cited for detailed procedures.

24.2 METHODS

24.2.1 Biochemical Oxygen Demand (BOD)

24.2.1.1 Principle

The BOD determination is a measure of the amount of oxygen required by microorganisms to oxidize the biodegradable organic constituents present in water and wastewater. The method is based on the direct relationship between the concentration of organic matter and the amount of oxygen used to oxidize the pollutants to water, carbon dioxide, and inorganic nitrogenous compounds. The oxygen demand of water and wastewater is proportional to the amount of organic matter present. The BOD method measures the biodegradable carbon (carbonaceous demand) and, under certain circumstances, the biodegradable nitrogen (nitrogeneous demand).

24.2.1.2 Procedure

Place a known amount of a water or wastewater sample that has been seeded with an effluent from a biological waste treatment plant in an airtight BOD bottle and measure the initial dissolved oxygen immediately. Incubate the sample at 20°C and, after 5 days, measure the dissolved oxygen content again (APHA Method 5210 B). The dissolved oxygen content can be determined by the membrane electrode method (APHA Method 4500-O G) or the azide modification of the iodometric method (APHA Method 4500-O C). The membrane electrode method is based on the diffusion rate of molecular oxygen across a membrane. A dissolved oxygen meter with an oxygen-sensitive membrane electrode is used to measure the diffusion current, which is linearly proportional to the concentration of molecular oxygen. The azide modified iodometric procedure is a titrimetric method that is based on the oxidizing property of dissolved oxygen. The BOD value, which is expressed as mg/liter, can be calculated from the difference in the initial dissolved oxygen and the content of dissolved oxygen after the incubation period according to the following equation (APHA Method 5210B):

$$BOD\ (mg/liter) = 100/P \times (DOB - DOD) \quad [1]$$

where:

DOB = initial oxygen in diluted sample, mg/liter

DOD = oxygen in diluted sample after 5-day incubation, mg/liter

P = ml sample × 100/capacity of bottle

24.2.1.3 Applications and Limitations

The BOD test is used most widely to measure the organic loading of waste treatment processes, to determine the efficiency of treatment systems, and to assess the effect of wastewater on the quality of receiving waters. The 5-day BOD test has some drawbacks because:

1. The procedure requires an incubation time of at least 5 days.
2. The BOD method does not measure all the organic materials that are biodegradable.
3. The test is not accurate without a proper seeding material.
4. Toxic substances such as chlorine present in water and wastewater may inhibit microbial growth.

24.2.2 Chemical Oxygen Demand (COD)

24.2.2.1 Principle

The COD determination is a rapid way to measure the quantity of oxygen used to oxidize the organic matter present in water and wastewater by a strong oxidizing agent. Most organic compounds are destroyed by refluxing in a strong acid solution with a known quantity of an oxidizing agent such as potassium dichromate. The excessive amount of potassium dichromate left after digestion of the organic matter is measured. The amount of organic matter that is chemically oxidizable is directly proportional to the potassium dichromate consumed.

24.2.2.2 Procedure

A known quantity of sample of water or wastewater is refluxed at elevated temperatures for up to 2 hr with a known quantity of potassium dichromate and sulfuric acid. The amount of potassium dichromate left after digestion of the organic matter is titrated with a standard ferrous ammonium sulfate (FAS) solution using orthophenanthroline ferrous complex as an indicator. The amount of oxidizable organic matter, determined as oxygen equivalent, is proportional to the potassium dichromate used in the oxidative reaction. The COD value can be calculated from the following equation (APHA Method 5220 B):

$$COD, \, mg/liter = (A - B) \times M \times 8000/D \quad [2]$$

where:

A = ml of FAS used for blank
B = ml of FAS used for sample
M = molarity of FAS
D = ml of sample used

24.2.2.3 Applications and Limitations

Potassium dichromate is widely used for the COD method because of its advantages over other oxidizing compounds in oxidizability, applicability to a wide variety of waste samples, and ease of manipulation. The dichromate reflux method can be used to measure the samples with COD values of greater than 50 mg/liter.

The COD test measures carbon and hydrogen in organic constituents but not nitrogenous compounds. Furthermore, the method does not differentiate between biologically stable and unstable compounds present in water and wastewater. The COD test is a very important procedure for routinely monitoring industrial wastewater discharges and for the control of waste treatment processes. The test is faster and more reproducible than the BOD method. The obvious disadvantages of the COD method are:

1. Aromatic hydrocarbons, pyridine, and straight-chain aliphatic compounds are not readily oxidized.
2. The method is very susceptible to interference by chloride, and thus the COD of certain food processing waste effluents such as pickle and sauerkraut brines cannot be readily determined without modification. This difficulty may be overcome by adding mercuric sulfate to the sample prior to refluxing. Chloride concentrations greater than 500–1000 mg/liter may not be corrected by the addition of mercuric sulfate. A chloride correction factor can be developed for a particular waste by the use of proper blanks.

24.2.3 Total Organic Carbon (TOC)

24.2.3.1 Principle

The TOC method is another rapid and convenient means for determining the amount of organic matter present in water and wastewater. The test uses high temperatures and a strong oxidizing agent to oxidize the organic carbon in water and wastewater to carbon dioxide and water. The carbon dioxide that is produced in the oxidative reaction is measured.

24.2.3.2 Procedure

Place a known quantity of a properly homogenized water or wastewater sample in an elevated-temperature reactor (900°C) containing an oxidizing agent such as cobalt oxide as a catalyst. Under controlled conditions, the carbon atoms of organic matter present in water and wastewater are converted to carbon dioxide and water. The amount of carbon dioxide produced in the oxidative reaction is determined quantitatively by means of an infrared total organic carbon analyzer (APHA Method 5310 B).

24.2.3.3 Applications and Limitations

The TOC test is especially useful for determining the amount of total organic carbon in water and wastewater. It is a rapid method and is more precise than COD and BOD. The major disadvantages of the TOC test are:

1. The test requires a well-trained technician and expensive equipment not normally found in a laboratory.
2. The TOC method does not completely oxidize all the organic carbon compounds present in water and wastewater.
3. TOC is generally not as reliable as BOD or COD in predicting the oxygen demand potential of water and wastewater because oxygen demands differ between organic compounds with the same number of organic carbons in their structure. For example, the oxygen demand of ethanol is six times greater than that of oxalic acid.

24.3 COMPARISON OF BOD, COD, AND TOC METHODS

The BOD and COD analyses of water and wastewater can result in different values because the two methods measure different materials. As shown in Table 24-1, the COD value of a waste sample is usually higher than its BOD and TOC values because:

24-1 table   Oxygen Demand of Tomato Processing Wastes

Item	1973	1974	1975
BOD, mg/liter	2,400	1,300	1,200
COD, mg/liter	5,500	3,000	2,800
TOC, mg/liter	2,000	1,100	1,000

From EPA. 1977. Pollution Abatement in the Fruit and Vegetable Industry. EPA-625/3-77-0007. Environmental Protection Agency, Washington, D.C

24-2 table   COD and BOD Values of Selected Fruit and Vegetable Processing Wastes

Product	COD (mg/liter)	BOD (mg/liter)	Mean Ratio (BOD/COD)
Apples	395–37,000	240–19,000	0.55
Beets	445–13,240	530–6400	0.57
Carrots	1750–2910	817–1927	0.52
Cherries	1200–3795	600–1900	0.53
Corn	3400–10,100	1587–5341	0.50
Green beans	78–2200	43–1400	0.55
Peas	723–2284	337–1350	0.61
Sauerkraut	470–65,000	300–41,000	0.66
Tomatoes	652–2305	454–1575	0.72
Wax beans	193–597	55–323	0.58
Wine	495–12,200	363–7645	0.60

From Splittstoesser, D.F., and Downing, D.L. 1969. Analysis of effluents from fruit and vegetable processing factories. N.Y. State Agr. Exp. Sta. Res. Circ. 17. Geneva, NY.

1. Many organic compounds that can be chemically oxidized cannot be biochemically oxidized. For example, cellulose cannot be determined by the BOD method but can be measured by the COD test.
2. Certain inorganic compounds such as ferrous iron, nitrites, sulfides, and thiosulfates are readily oxidized by potassium dichromate. This inorganic COD introduces an error when computing the organic matter of water and wastewater.
3. The BOD test can give low values because of a poor seeding material. The COD test does not requires an inoculum.
4. Some aromatics and nitrogenous (ammonium) compounds are not oxidized by the COD method. Other organic constituents such as cellulose or lignin, which are readily oxidized by potassium dichromate, are not biologically degraded by the BOD method.
5. Toxic materials present in water and wastewater that do not interfere with the COD test can affect the BOD results.

The COD, however, has value for specific wastes since it is possible to obtain a direct correlation between COD and BOD values. Table 24-2 shows the COD and BOD values of waste effluents from fruit and vegetable processing factories. The BOD/COD ratios of these processing waste effluents varied considerably and ranged from 0.50 to 0.72 (Splitstoesser and Downing, 1969). The BOD/COD ratio can be a useful tool for rapid determination of the biodegradability of organic matter present in the wastes. A low BOD/COD ratio indicates the presence of a large amount of nonbiodegradable organic matter. Samples of wastewater with high BOD/COD ratios have a small amount of organic matter that is nonbiodegradable.

The TOC test is a rapid and convenient means to estimate BOD or COD, once an empirical relationship between TOC and BOD or TOC and COD has been established. The TOC method may give a lower value than the actual amount of organic matter present in

water and wastewater because certain organic matter may not be oxidized completely (Table 24-1). Furthermore, samples of water and wastewater that contain certain inorganic components, such as carbonates and bicarbonates, may give slightly higher TOC values.

24.4 SAMPLING AND HANDLING REQUIREMENTS

Samples of water and wastewater collected for oxygen demand determinations must be analyzed as soon as possible or stored under properly controlled conditions until analyses can be made.

Samples for the BOD test can be kept at low temperatures (4°C or below) for up to 48 hr. Chemical preservatives should not be added to water and wastewater because they can interfere with BOD analysis.

Untreated wastewater samples for the COD test must be collected in glass containers and analyzed promptly. The COD samples can be stored at 4°C or below for up to 28 days if these are acidified with a concentrated mineral acid (sulfuric acid) to a pH value of 2.0 or below.

Samples of water and wastewater for the TOC test must be collected in amber glass containers. If the test cannot be made immediately, the TOC samples must be kept in the dark at low temperatures (4°C or below) or they can be preserved by acidification to pH 2.0 or below using concentrated sulfuric or phosphoric acid.

24.5 SUMMARY

Oxygen demand is most widely used to determine the effect of organic pollutants present in water and wastewater on receiving streams and rivers. The three

important methods used to measure oxygen demand are BOD, COD, and TOC.

The BOD test measures the amount of oxygen required by microorganisms to oxidize the biodegradable organic matter present in water and wastewater. The COD method determines the quantity of oxygen consumed during the oxidation of organic matter in water and wastewater by potassium dichromate. The TOC determination is a measure of the amount of total organic carbon present in water and wastewater that can be converted to carbon dioxide and water by means of a strong oxidizing agent such as potassium dichromate at elevated temperatures.

Of the three methods used to measure oxygen demand, the BOD test has the widest application in measuring waste loading to treatment systems, in determining the efficiency of treatment processes, and in evaluating the quality of receiving streams and rivers because it most closely approximates the natural conditions of the environment. The COD or TOC test can be used to monitor routinely the biodegradability of organic matter in water and wastewater if a relationship between COD and BOD or TOC and BOD has been established.

24.6 STUDY QUESTIONS

1. Differentiate the principles of the BOD, COD, and TOC methods to measure oxygen demand.
2. In your new job as supervisor of a lab that has previously been using the BOD method to determine oxygen demand of wastewater, you have decided to change to the COD method.
   a. Describe the basic principle and procedure of the COD method to your lab technicians.
   b. In what case would they be instructed to use mercuric sulfate in the COD assay?
   c. You realize there are advantages and disadvantages of all three potential methods—BOD, COD, and TOC. Give two advantages and two disadvantages for each

of the BOD and TOC methods as compared to the COD method.
3. In each case described below, indicate if you would expect the COD value to be higher or lower than results from a BOD test. Explain your answer.
   a. poor seed material in BOD test
   b. sample contains toxic materials
   c. sample high in aromatics and nitrogenous compounds
   d. sample high in nitrites and ferrous iron
   e. sample high in cellulose and lignin

24.7 PRACTICE PROBLEMS

1. Determine the BOD value of a sample given the following data (see Equation [1]):
   DOB = 9.0 mg/liter
   DOD = 6.6 mg/liter
   P = 15 ml
   Capacity of bottle = 300 ml
2. Determine the COD value of a sample given the following data (see Equation [2]):
   FAS for blank = 37.8 ml
   FAS for sample = 34.4 ml
   molarity of FAS = 0.025 M
   sample = 5 ml

Answers

1. BOD = 48 mg/liter; 2. COD = 136 mg/liter.

24.8 RESOURCE MATERIALS

EPA. 1977. Pollution Abatement in the Fruit and Vegetable Industry. EPA-625/3-77-0007. Environmental Protection Agency, Washington, DC.

Eaton, A.D., Clesceri, L.S., and Greenberg, A.E. (Eds.) 1995. *Standard Methods for the Examination of Water and Wastewater*, 19th ed. American Public Health Association (APHA), Washington, DC.

Splittstoesser, D.F., and Downing, D.L. 1969. Analysis of effluents from fruit and vegetable processing factories. N.Y. State Agr. Exp. Sta. Res. Circ. 17. Geneva, NY.

part III

Spectroscopy

Basic Principles of Spectroscopy

Michael H. Penner

25.1 INTRODUCTION

Spectroscopy deals with the production, measurement, and interpretation of spectra arising from the interaction of electromagnetic radiation with matter. There are many different spectroscopic methods available for solving a wide range of analytical problems. The methods differ with respect to the species to be analyzed (such as molecular or atomic spectroscopy), the type of radiation–matter interaction to be monitored (such as absorption, emission, or diffraction), and the region of the electromagnetic spectrum used in the analysis. Spectroscopic methods are very informative and widely used for both quantitative and qualitative analyses. Spectroscopic methods based on the absorption or emission of radiation in the ultraviolet (UV), visible (Vis), infrared (IR), and radio (nuclear magnetic resonance, NMR) frequency ranges are most commonly encountered in traditional food analysis laboratories. Each of these methods is distinct in that it monitors different types of molecular or atomic transitions. The basis of these transitions is explained in the following sections.

25.2 LIGHT

25.2.1 Properties

Light may be thought of as particles of energy that move through space with wavelike properties. This image of light suggests that the energy associated with a ray of light is not distributed continuously through space along the wave's associated electric and magnetic fields but rather that it is concentrated in discrete packets. Light is therefore said to have a dual nature: particulate and wavelike. Phenomena associated with light propagation, such as interference, diffraction, and refraction, are most easily explained using the wave theory of electromagnetic radiation. However, the interaction of light with matter, which is the basis of absorption and emission spectroscopy, may be best understood in terms of the particulate nature of light. Light is not unique in possessing both wavelike and particulate properties. For example, fundamental particles of matter, such as electrons, protons, and neutrons, are known to exhibit wavelike behavior.

The wave properties of electromagnetic radiation are described in terms of the wave's frequency, wavelength, and amplitude. A graphical representation of a plane-polarized electromagnetic wave is given in Fig. 25-1. The wave is plane polarized in that the oscillating electric and magnetic fields making up the wave are each limited to a single plane. The frequency (v) of a wave is defined as the number of oscillations the wave will make at a given point per second. This is the reciprocal of the period (p) of a wave, which is the time in seconds required for successive maxima of the wave to

Electric field (E)
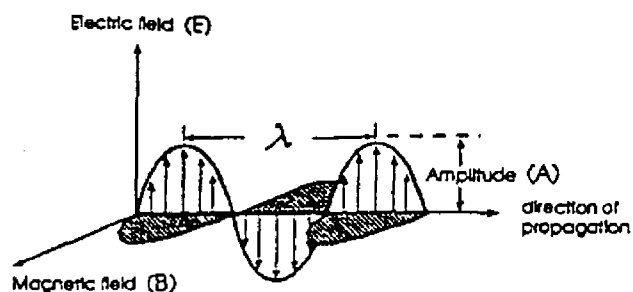
Magnetic field (B)

25-1
figure
Representation of plane-polarized electromagnetic radiation propagating along the x-axis. The electric and magnetic fields are in phase, perpendicular to each other and to the direction of propagation.

pass a fixed point. The wavelength (λ) represents the distance between successive maxima on any given wave. The units used in reporting wavelengths will depend on the region of electromagnetic radiation used in the analysis. Spectroscopic data sometimes are reported with respect to wavenumbers ($\bar{v}$), which are reciprocal wavelengths in units of cm^{-1}. Wavenumbers are encountered most often in infrared spectroscopy. The velocity of propagation (v_i) of an electromagnetic wave, in units of distance per second, in any given medium "i" can be calculated by taking the product of the frequency of the wave, in cycles per second, and its wavelength in that particular medium.

$$\bar{v}_i = v\lambda_i \qquad [1]$$

where:

v_i = velocity of propagation in medium "i"
v = wave frequency
λ_i = wavelength in medium "i"

The frequency of an electromagnetic wave is determined by the source of the radiation, and it remains constant as the wave traverses different media. However, the velocity of propagation of a wave will vary slightly depending on the medium through which the light is propagated. The wavelength of the radiation will change in proportion to changes in wave velocity as defined by Equation [1]. The amplitude of the wave (A) represents the magnitude of the electric vector at the wave maxima. The radiant power (P) and radiant intensity (I) of a beam of radiation are proportional to the square of the amplitude of the associated waves making up that radiation. Figure 25-1 indicates that electromagnetic waves are composed of oscillating magnetic and electric fields, the two of which are mutually perpendicular, in phase with each other, and perpendicular to the direction of wave propagation. As drawn, the waves represent changes in the respective field strengths with time at a fixed location or changes in the respective field strengths over distance at a fixed

time. The electrical and magnetic components of the waves are represented as a series of vectors whose lengths are proportional to the magnitude of the respective field. It is the oscillating electric field that is of most significance to spectroscopic phenomena such as absorption, transmission, and refraction. However, a purely electric field, without its associated magnetic field, is impossible.

22.2.2 Terminology

The propagation of electromagnetic waves is often described in terms of wavefronts or trains of waves (Fig. 25-2). A wavefront represents the locus of a set of points all of which are in phase. For a point source of light, a concentric ring that passes through the maxima of adjacent light rays will represent a wavefront. The entire ring need not be drawn in all cases, such that wavefronts may represent planes of light in cases where the observation is sufficiently removed from the point source that the curved surface appears planer. Wavefronts are most typically drawn by connecting maxima, minima, or both for adjacent rays. If maxima are used for depicting wavefronts, then each of the wavefronts will be separated by one wavelength. A train of waves, or wave-train, refers to a series of wavefronts all of which are in phase, that is, each individual wave will have a maximum amplitude at the same location in space. A wave-train also may be represented by a series of light rays. Rays of light are used generally with reference to the corpuscular nature of light, representing the path of photons. A wave-train would indicate that a series of photons, all in phase, followed the same path.

25.2.3 Interference

Interference is the term used to describe the observation that when two or more wave-trains cross one another, they result in an instantaneous wave, at the point of intersection, whose amplitude is the algebraic sum of the amplitudes of the individual waves at the point of intersection. The law describing this wave
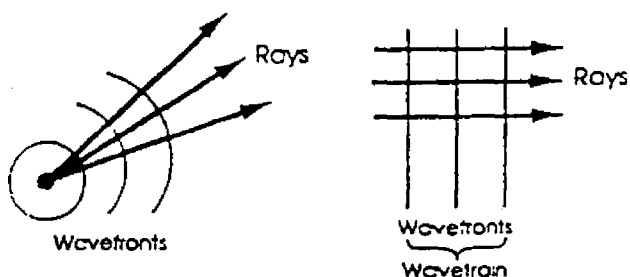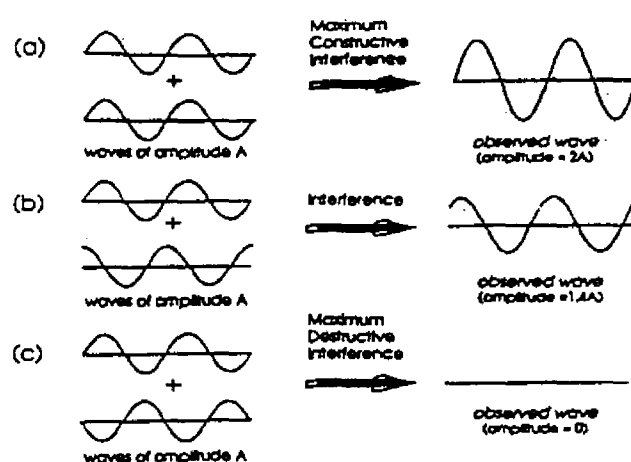
25-3 figure Interference of identical waves that are (a) in phase, (b) 90° out of phase, and (c) 180° out of phase.

behavior is known as the principle of superposition. Superposition of sinusoidal waves is illustrated in Fig. 25-3. Note that in all cases, the effective amplitude of the perceived wave at the point in question is the combined effect of each of the waves that crosses that point at any given instant. In spectroscopy, the amplitude of most general interest is that corresponding to the magnitude of the resulting electric field intensity. Maximum constructive interference of two waves occurs when the waves are completely in phase (i.e., the maxima of one wave aligns with the maxima of the other wave), while maximum destructive interference occurs when waves are 180' out of phase (the maxima of one wave aligns with the minima of the other wave). This concept of interference is fundamental to the interpretation of diffraction data, which represents a specialized segment of qualitative spectroscopy. Interference phenomena also are widely used in the design of spectroscopic instruments that require the dispersion or selection of radiation, such as those instruments employing grating monochromators or interference filters, as described in Chapter 26.

Interference phenomena are best rationalized by considering the wavelike nature of light. However, phenomena such as the absorption and emission of radiation are more easily understood by considering the particulate nature of light. The particles of energy that move through space with wavelike properties are called photons. The energy of a photon can be defined in terms of the frequency of the wave with which it is associated (Equation [2]).

$$E = h\nu \qquad [2]$$

where:

E = energy of a photon

h = Planck's constant

v = frequency of associated wave

This relationship indicates that the photons making up monochromatic light, which is electromagnetic radiation composed of waves having a single frequency and wavelength, are all of equivalent energy. Furthermore, just as the frequency of a wave is a constant determined by the radiation source, the energy of associated photons also will be unchanging. The brightness of a beam of monochromatic light, when expressed in terms of the particulate nature of light, will be the product of the photon flux and the energy per photon. The photon flux refers to the number of photons flowing across a unit area perpendicular to the beam per unit time. It follows that to change the brightness of a beam of monochromatic light will require a change in the photon flux. In spectroscopy, the term *brightness* is generally not used, but rather one refers to the radiant power (P) or the radiant intensity (I) of a beam of light. Radiant power and radiant intensity often are used synonymously when referring to the amount of radiant energy striking a given area per unit time. In terms of SI Units (time, seconds; area, meters^2; energy, joules), radiant power equals the number of joules of radiant energy impinging on a 1 meter^2 area of detector per second. The basic interrelationships of light-related properties and a general scheme of the electromagnetic spectrum are presented in Table 25-1 and Fig. 25-4, respectively.

25.3 ENERGY STATES OF MATTER

25.3.1 Quantum Nature of Matter

The energy content of matter is quantized. Consequently, the potential or internal energy content of an

atom or molecule does not vary in a continuous manner but rather in a series of discrete steps. Atoms and molecules, under normal conditions, exist predominantly in the ground state, which is the state of lowest energy. Ground state atoms and molecules can gain energy, in which case they will be elevated to one of their higher energy states, referred to as excited states. The quantum nature of atoms and molecules puts limitations on the energy levels that are available to these species. Consequently, there will be specific "allowed" internal energy levels for each atomic or molecular species. Internal energy levels not corresponding to an allowed value for that particular species are unattainable. The set of available energy levels for any given atom or molecule will be distinct for that species. Similarly, the potential energy spacings between allowed internal energy levels will be characteristic of a species. Therefore, the set of potential energy spacings for a species may be used qualitatively as a distinct fingerprint. Qualitative absorption and emission spectroscopy make use of this phenomenon in that these techniques attempt to determine an unknown compound's relative energy spacings by measuring transitions between allowed energy levels.

25.3.2 Electronic, Vibrational, and Rotational Energy Levels

The relative potential energy of an atom or molecule corresponds to the energy difference between the energy state in which the species exists and that of the ground state. Figure 25-5 is a partial molecular energy level diagram depicting potential energy levels for an organic molecule. The lowest energy state in the figure, bottom line in bold, represents the ground state. There are three electronic energy states depicted, each with

25-1 table   Properties of Light

Symbols/Terms	Relationship	Frequently Used Units
λ = wavelength	$\lambda v = c$	nm (nanometers, 10^{-9} m) Å (Ångstrom units, 10^{-10} m) μm (microns, 10^{-6} m) mμ (millimicrons, 10^{-9} m)
v = frequency c = speed of light $\bar{v}$ = wavenumber	= $1/\lambda$	Hz (hertz, 1 Hz = 1 oscillation per second) sec^{-1} 2.9979 x 10^8 m sec^{-1} in vacuum cm^{-1} kK (kilokayser, 1 kK = 1000 cm^{-1})
p = period E = energy	$p = 1/\lambda$ $E = hv$ = hc/λ = $hc\bar{v}$	seconds J (1 joule = 1 kg m^2 sec^{-2}) cal (calorie, 1 cal = 4.184 J) erg (1 erg = 10^{-7} J) eV (1 electron volt = 1.6022 x 10^{-19} J)
h = Planck's constant P = radiant power	amount of energy striking a given unit area per unit time	6.6262 x 10^{-34} J sec (Joules)(m^2)$^{-1}$ (sec)$^{-1}$

Wavelength (λ) In meters		Frequency (ν) In waves per second	Energy ($E=h\nu$) In kilocalories/mole
	cosmic rays	-3×10^{-13} 10^{20}	10^{12}
	gamma rays	-3×10^{-11} 10^{19}	10^{11}
		-3×10^{-10} 10^{18}	10^{10}
380 nm violet	x-rays	-3×10^{-8} 10^{17}	10^{9}
450 nm blue	ultraviolet	-3×10^{-8} 10^{16}	10^{8}
500 nm blue green		-3×10^{-7} 10^{15}	10^{7}
550 nm green yellow		-3×10^{-6} 10^{14}	10^{6}
600 nm orange	near infrared	-3×10^{-5} 10^{13}	10^{5}
650 nm red			
720 nm	far infrared	-3×10^{-4} 10^{12}	10^{4}
		-3×10^{-3} 10^{11}	10^{3}
		-3×10^{-2} 10^{10}	10^{2}
	microwaves	-3×10^{-1} 10^{9}	10^{1}
	police radio FM radio	-3×10^{0} 10^{8}	10^{0}
	TV broadcast	-3×10^{1} 10^{7}	10^{-1}
	AM radio	-3×10^{2} 10^{6}	10^{-2}
		-3×10^{3} 10^{5}	10^{-3}
		-3×10^{4} 10^{4}	10^{-4}

Wave   Quantum

25-4 figure   The electromagnetic spectrum. Milton Roy Education Manual, 1989, p. 3. (Courtesy of Milton Roy Company, Rochester, NY, a subsidiary of Sundstrand Corporation.)

its corresponding vibrational and rotational energy levels. Each of the electronic states corresponds to a given electron orbital. Electrons in different orbitals are of different potential energy. When an electron changes orbitals, such as when absorbing or emitting a photon of appropriate energy, it is termed an electronic transition since it is the electron that is changing energy levels. However, any change in the potential energy of an electron will, by necessity, result in a corresponding change in the potential energy of the atom or molecule that the electron is associated with.

Atoms are like molecules in that only specific energy levels are allowed for atomic electrons. Consequently, an energy level diagram of an atom would consist of a series of electronic energy levels. In contrast to molecules, the electronic energy levels of atoms have no corresponding vibrational and rotational levels and, hence, may appear less complicated. Atomic energy levels correspond to allowed electron shells (orbits) and corresponding subshells (i.e., 1s, 2s, 2p, etc.). The magnitude of the energy difference between the ground state and first excited states for valence electrons of atoms and bonding electrons of molecules is

generally of the same range as the energy content of photons associated with UV and Vis radiation.

The wider lines within each electronic state of Fig. 25-5 depict the species' vibrational energy levels. The atoms that comprise a molecule are in constant motion, vibrating in many ways. However, in all cases the energy associated with this vibrational motion corresponds to defined quantized energy levels. The energy differences between neighboring vibrational energy levels are much smaller than those between adjacent electronic energy levels. Therefore, it is common to consider that several vibrational energy levels are superimposed on each of the molecular electronic energy levels. Energy differences between allowed vibrational energy levels are of the same magnitude as the energy of photons associated with radiation in the infrared region. Vibrational energy levels would not be superimposed on an atomic potential energy level diagram since this vibrational motion does not exist in a single atom. In this respect,the potential energy diagram for an atom is less complex than that for a molecule, the atomic energy level diagram having fewer energy levels.
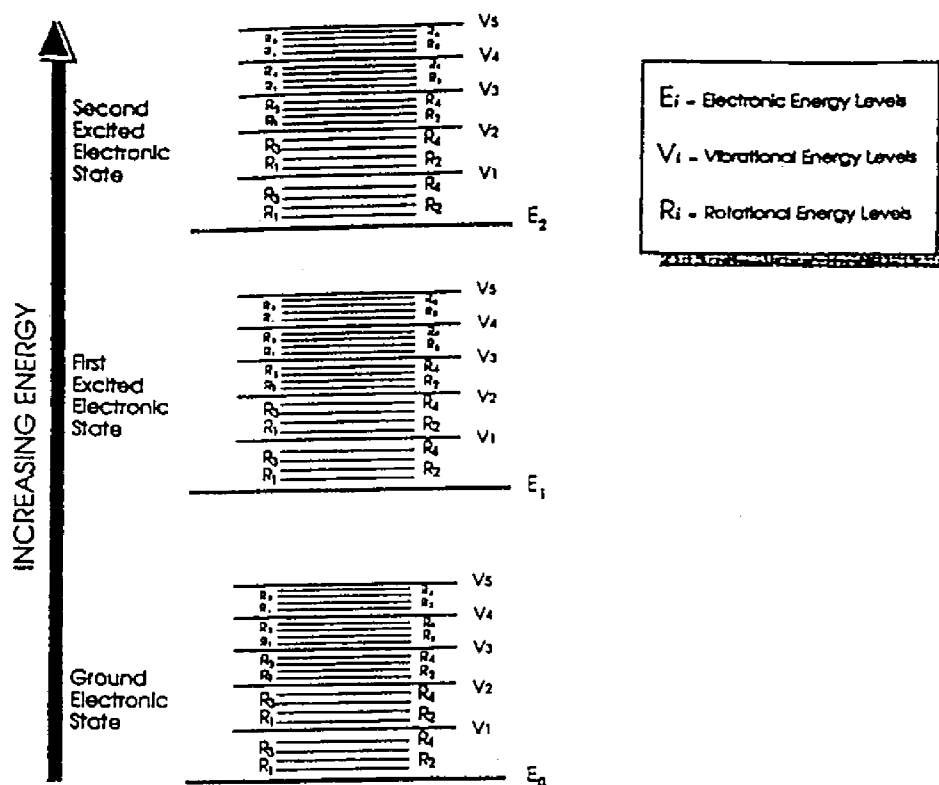
25-5 figure Partial molecular energy level diagram depicting three electronic states.

The potential energy of a molecule also is quantized in terms of the energy associated with the rotation of the molecule about its center of gravity. These rotational energy levels are yet more closely spaced than the corresponding vibrational levels, as depicted by the narrow lines within each electronic state shown in Fig. 25-5. Hence it is customary to consider several rotational energy levels superimposed on each of the permitted vibrational energy levels. The energy spacings between rotational energy levels are of the same magnitude as the energy associated with photons of microwave radiation. Microwave spectroscopy is not commonly used in food analysis laboratories; however, the presence of these different energy levels will impact the spectrum observed in other forms of spectroscopy, as will be discussed later. Similar to the situation of vibrational energy levels, rotational energy levels are not of consequence to atomic spectroscopy.

In summation, the internal energy of an atom is described in terms of its electronic energy levels, while the internal energy of a molecule is dependent on its electronic, vibrational, and rotational energies. The algebraic form of these statements follows.

$$E_{atom} = E_{electronic} \qquad [3]$$

$$E_{molecule} = E_{electronic} + E_{vibrational} + E_{rotational} \qquad [4]$$

The spectroscopist makes use of the fact that each of these associated energies is quantized and that different species will have somewhat different energy spacings.

25.3.3 Nuclear Energy Levels in Applied Magnetic Fields

Nuclear magnetic resonance (NMR) spectroscopy makes use of yet another type of quantized energy level. The energy levels of importance to NMR spectroscopy differ with respect to those described above in that they are relevant only in the presence of an applied external magnetic field. The basis for the observed energy levels may be rationalized by considering that the nuclei of some atoms behave as tiny bar magnets. Hence, when the atoms are placed in a magnetic field, their nuclear magnetic moment will have a preferred orientation, just as a bar magnet would behave. The NMR-sensitive nuclei of general relevance to the food analyst have two permissible orientations. The energy difference between these allowed orientations depends on the effective magnetic field strength that the nuclei experience. The effective magnetic field strength will itself depend on the strength of the applied magnetic field and the chemical environment surrounding the nuclei in question. The applied magnetic field strength

will be set by the spectroscopist, and it is essentially equivalent for each of the nuclei in the applied field. Hence, differences in energy spacings of NMR-sensitive nuclei will depend solely on the identity of the nucleus and its environment. In general, the energy spacings between permissible nuclear orientations, under usable external magnetic field strengths, are of the same magnitude as the energy associated with radiation in the radiofrequency range.

25.4 ENERGY LEVEL TRANSITIONS IN SPECTROSCOPY

25.4.1 Absorption of Radiation

The absorption of radiation by an atom or molecule is that process in which energy from a photon of electromagnetic radiation is transferred to the absorbing species. When an atom or molecule absorbs a photon of light, its internal energy increases by an amount equivalent to the amount of energy in that particular photon. Therefore, in the process of absorption, the species goes from a lower energy state to a more excited state. In most cases, the species is in the ground state prior to absorption. Since the absorption process may be considered quantitative, i.e., all of the photon's energy is transferred to the absorbing species, the photon being absorbed must have an energy content that exactly matches the energy difference between the energy levels across which the transition occurs. This must be the case due to the quantized energy levels of matter, as discussed previously. Consequently, if one plots photon energy versus the relative absorbance of radiation uniquely composed of photons of that energy, one observes a characteristic absorption spectrum, the shape of which is determined by the relative absorbtivity of photons of different energy. The absorbtivity of a compound is a wavelength-dependent proportionality constant that relates the absorbing species concentration to its experimentally measured absorbance under defined conditions. A representative absorption spectrum covering a portion of the UV radiation range is presented in Fig. 25-6. The independent variable of an absorption spectrum is most commonly expressed in terms of the wave properties (wavelength, frequency, or wavenumbers) of the radiation, as in Fig. 25-6, rather than the energy of the associated photons.

Various molecular transitions resulting from the absorption of photons of different energy are shown schematically in Fig. 25-7. The transitions depicted represent those that may be induced by absorption of UV, Vis, IR, and microwave radiation. The figure also includes transitions in which the molecule is excited from the ground state to an exited electronic state with a simultaneous change in its vibrational or rotational energy levels. Although not shown in the figure, the

WAVELENGTH (nm)
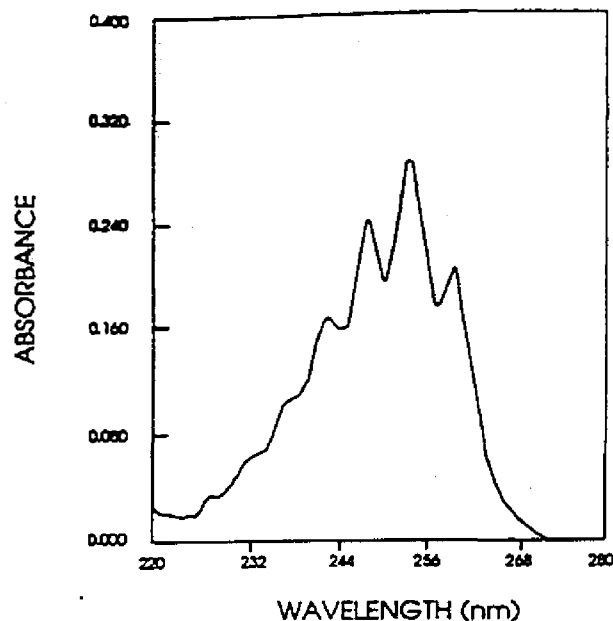
25-6 Absorption spectrum of a 0.005 M benzene in water solution.
figure
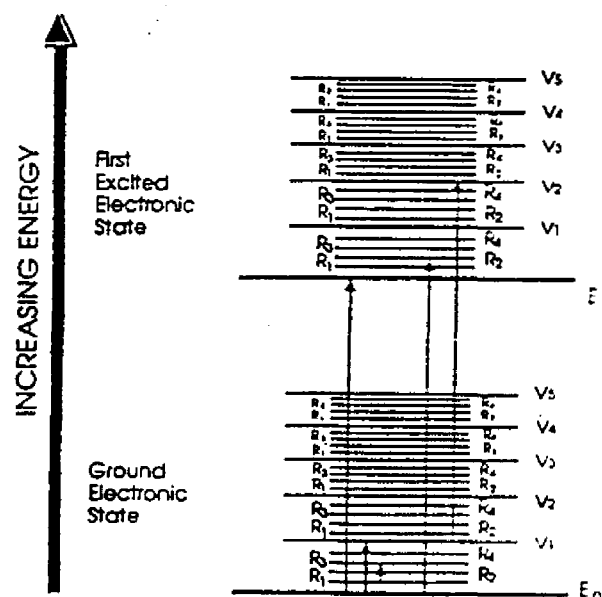
25-7 Partial molecular energy level diagram including electronic, vibrational, and rotational transitions.
figure

absorption of a photon of appropriate energy also may cause simultaneous changes in electronic, vibrational, and rotational energy levels. The ability of molecules to have simultaneous transitions between the different energy levels tends to broaden the peaks in the UV-Vis absorption spectrum of molecules relative to those peaks observed in the absorption spectrum of atoms.

This would be expected when one considers that vibrational and rotational energy levels are absent in an atomic energy level diagram. The depicted transitions between vibrational energy levels, without associated electronic transitions, are induced by radiation in the infrared region. Independent transitions between allowed rotational energy levels also are depicted, these resulting from the absorption of photons of microwave radiation. A summary of transitions relevant to atomic and molecular absorption spectroscopy, including corresponding wavelength regions, is presented in Table 25-2.

25.4.2 Emission of Radiation

Emission is essentially the reverse of the absorption process, occurring when energy from an atom or molecule is released in the form of a photon of radiation. A molecule raised to an excited state will typically remain in the excited state for a very short period of time before relaxing back to the ground state. There are several relaxation processes through which an excited molecule may dissipate energy. The most common relaxation process is for the excited molecule to dissipate its energy through a series of small steps brought on by collisions with other molecules. The energy is thus converted to kinetic energy, the net result being the dissipation of the energy as heat. Under normal conditions, the dissipated heat is not enough to measurably affect the system. In some cases, molecules excited by the absorption of UV or Vis light will lose a portion of their excess energy through the emission of a photon. This emission process is referred to as either

fluorescence or phosphorescence, depending on the nature of the excited state. In molecular fluorescence spectroscopy, the photons emitted from the excited species generally will be of lower energy and longer wavelength than the corresponding photons that were absorbed in the excitation process. The reason is that, in most cases, only a fraction of the energy difference between the excited and ground states is lost in the emission process. The other fraction of the excess energy is dissipated as heat during vibrational relaxation. This process is depicted in Fig. 25-8, which illustrates that the excited species undergoes vibrational relaxation down to the lowest vibrational energy level within the excited electronic state, and then undergoes a transition to the ground electronic state through the emission of a photon. The photon emitted will have an energy that equals the energy difference between the lowest vibrational level of the excited electronic state and the ground electronic state level it descends to. The fluorescing molecule may descend to any of the vibrational levels within the ground electronic state. If the fluorescence transition is to an excited vibrational level within the ground electronic state, then it will quickly return to the ground state (lowest energy level) via vibrational relaxation. In yet other cases, an excited species may be of sufficient energy to initiate some type of photochemistry that ultimately leads to a decrease in the system's potential energy. In all cases, the relaxation process is driven by the tendency for a species to exist at its lowest permissible internal energy level. The relaxation process that dominates a system will be the one that minimizes the lifetime of the excited state. Under normal conditions, the relaxation

25-2 table

Wavelength Regions, Spectroscopic Methods, and Associated Transitions

Wavelength Region	Wavelength Limits	Type of Spectroscopy	Usual Wavelength Range	Types of Transitions in Chemical Systems with Similar Energies
Gamma rays	0.01–1 Å	Emission	<0.1 Å	Nuclear proton/neutron arrangements
X-rays	0.1–10 nm	Absorption, emission, fluorescence, and diffraction	0.1–100 Å	Inner-shell electrons
Ultraviolet	10–380 nm	Absorption, emission, and fluorescence	180–380 nm	Outer-shell electrons in atoms, bonding electrons in molecules
Visible	380–750 nm	Absorption, emission, and fluorescence	380–750 nm	Same as ultraviolet
Infrared	0.075–1000 μm	Absorption	0.78–300 μm	Vibrational position of atoms in molecular bonds
Microwave	0.1–100 cm	Absorption	0.75–3.75 mm	Rotational position in molecules
		Electron spin resonance	3 cm	Orientation of unpaired electrons in an applied magnetic field
Radiowave	1–1000 m	Nuclear magnetic resonance	0.6–10 m	Orientation of nuclei in an applied magnetic field

INCREASING ENERGY

First Excited Electronic State

E_1

Ground Electronic State

E_0
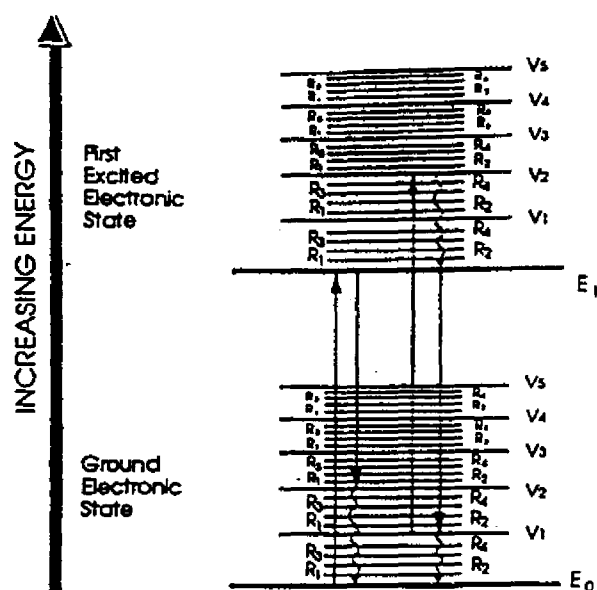
25-8 figure Partial molecular energy level diagram including absorption, vibrational relaxation, and fluorescence relaxation.

process is so rapid that the population of molecules in the ground state is essentially unchanged.

25.5 SUMMARY

Spectroscopy deals with the interaction of electromagnetic radiation with matter. Spectrochemical analysis, a branch of spectroscopy, encompasses a wide range of techniques used in analytical laboratories for the qualitative and quantitative analysis of the chemical composition of foods. Common spectrochemical analysis methods include ultraviolet, visible, and infrared absorption spectroscopy; molecular fluorescence spectroscopy; and nuclear magnetic resonance spectroscopy. In each of these methods, the analyst attempts to measure the amount of radiation either absorbed or emitted by the analyte. All of these methods make use of the facts that the energy content of matter is quantized and that photons of radiation may be absorbed or emitted by matter if the energy associated with the photon equals the energy difference for allowed transitions of that given species. The above methods differ from each other with respect to the radiation wavelengths used in the analysis or the molecular versus atomic nature of the analyte.

25.6 STUDY QUESTIONS

1. Which phenomena associated with light are most readily explained by considering the wave nature of light? Explain these phenomena based on your understanding of interference.

2. Which phenomena associated with light are most readily explained by considering the particulate nature of light? Explain these phenomena based on your understanding of the quantum nature of electromagnetic radiation.

3. What does it mean to say that the energy content of matter is quantized?

4. Molecular absorption of radiation in the UV-Vis range results in transitions between what types of energy levels?

5. Molecular absorption of radiation in the IR range results in transitions between what types of energy levels?

6. Why is an applied magnetic field necessary for NMR spectroscopy?

7. How do the allowed energy levels of molecules differ from those of atoms? Answer with respect to the energy level diagram depicted in Fig. 25-5.

8. In fluorescence spectroscopy, why is the wavelength of the emitted radiation longer than the wavelength of the radiation used for excitation of the analyte?

25.7 RESOURCE MATERIALS

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Ultraviolet, Visible, and Fluorescence Spectroscopy

Michael H. Penner

26.1 INTRODUCTION

Spectroscopy in the ultraviolet-visible (UV-Vis) range is one of the most commonly encountered laboratory techniques in food analysis. Electromagnetic radiation in the UV-Vis portion of the spectrum ranges in wavelength from approximately 200 to 700 nm. The UV range runs from 200 to 350 nm and the Vis range from 350 to 700 nm (Table 23-1). The UV range is colorless to the human eye, while different wavelengths in the visible range each have a characteristic color, ranging from violet at the short wavelength end of the spectrum to red at the long wavelength end of the spectrum. Spectroscopy utilizing radiation in the UV-Vis range may be divided into two general categories, absorbance and fluorescence spectroscopy, based on the type of radiation—matter interaction that is being monitored. Each of these two types of spectroscopy may be subdivided further into qualitative and quantitative techniques. In general, quantitative absorption spectroscopy is the most common of the subdivisions within UV-Vis spectroscopy.

26.2 ULTRAVIOLET AND VISIBLE ABSORPTION SPECTROSCOPY

26.2.1 Basis of Quantitative Absorption Spectroscopy

The objective of quantitative absorption spectroscopy is to determine the concentration of analyte in a given sample solution. The determination is based on the measurement of the amount of light absorbed from a reference beam as it passes through the sample solution. In some cases, the analyte may naturally absorb radiation in the UV-Vis range, such that the chemical nature of the analyte is not modified during the analysis. In other cases, analytes that do not absorb radiation in the UV-Vis range are chemically modified during the analysis, converting them to a species that absorbs radiation of the appropriate wavelength. In either case, the presence of analyte in the solution will affect the amount of radiation transmitted through the solution and, hence, the relative transmittance or absorbance of the solution may be used as an index of analyte concentration.

In actual practice, the solution to be analyzed is contained in an absorption cell and placed in the path of radiation of a selected wavelength(s). The amount of radiation passing through the sample then is measured relative to a reference sample. The relative amount of light passing through the sample is then used to estimate the analyte concentration. The process of absorption may be depicted as in Fig. 26-1. The radiation incident on the absorption cell, P_0, will have significantly greater radiant power than the radiation exiting the opposite side of the cell, P. The decrease in radiant power as the beam passes through the solution is due to the capture (absorption) of photons by the absorbing species. The relationship between the power of the incident and exiting beams typically is expressed in terms of either the transmittance or the absorbance of the solution. The transmittance (T) of a solution is defined as the ratio of P to P_0 as given in Equation [1]. Transmittance also may be expressed as a percentage as given in Equation [2].

$$T = P/P_0 \qquad [1]$$

$$\%T = (P/P_0) \times 100 \qquad [2]$$

where:

T = transmittance
P_0 = radiant power of beam incident on absorption cell

26-1 **table** Spectrum of Visible Radiation

Wavelength (nm)	Color	Complementary Hue[1]
<380	Ultraviolet	
380–420	Violet	Yellow-green
420–440	Violet-blue	Yellow
440–470	Blue	Orange
470–500	Blue-green	Red
500–520	Green	Purple
520–550	Yellow-green	Violet
550–580	Yellow	Violet-blue
580–620	Orange	Blue
620–680	Red	Blue-green
680–780	Purple	Green
>780	Near-infrared	

[1]Complementary hue refers to the color observed for a solution that shows maximum absorbance at the designated wavelength assuming a continuous spectrum "white" light source
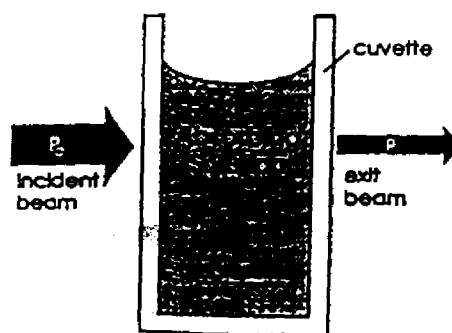
26-1 **figure** Attenuation of a beam of radiation as it passes through a cuvette containing an absorbing solution.

P = radiant power of beam exiting the absorption cell

$\%T$ = percent transmittance

The terms T and $\%T$ are intuitively appealing, as they express the fraction of the incident light absorbed by the solution. However, T and $\%T$ are not directly proportional to the concentration of the absorbing analyte in the sample solution. The nonlinear relationship between transmittance and concentration is an inconvenience since analysts are generally interested in analyte concentrations. A second term used to describe the relationship between P and P_0 is absorbance (A). Absorbance is defined with respect to T as shown in Equation [3].

$$A = \log (P_0/P) = -\log T = 2 - \log \%T \qquad [3]$$

where:

A = absorbance

T, $\%T$ as in Equations [1] and [2], respectively

Absorbance is a convenient expression in that, under appropriate conditions, it is directly proportional to the concentration of the absorbing species in the solution. Note that, based on these definitions for A and T, the absorbance of a solution is *not* simply unity minus the transmittance. In quantitative spectroscopy, the fraction of the incident beam that is not transmitted does not equal the solution's absorbance (A).

The relationship between the absorbance of a solution and the concentration of the absorbing species is known as Beer's law (Equation [4]).

$$A = abc \qquad [4]$$

where:

A = absorbance

c = concentration of absorbing species

b = path length through solution (cm)

a = absorptivity

There are no units associated with absorbance, A, since it is the log of a ratio of beam powers. The concentration term, c, may be expressed in any appropriate units (M, mM, mg/ml, %). The path length, b, is in units of cm. The absorptivity, a, of a given species is a proportionality constant dependent on the molecular properties of the species. The absorptivity is wavelength dependent and may vary depending on the chemical environment (pH, ionic strength, solvent, etc.) the absorbing species is experiencing. The units of the absorptivity term are $(cm)^{-1}$ (concentration)$^{-1}$. In the special case where the concentration of the analyte is reported in units of molarity, the absorptivity term has units of $(cm)^{-1} (M)^{-1}$. Under these conditions, it is des-

ignated by the symbol ϵ, which is referred to as the molar absorptivity. Beer's law expressed in terms of the molar absorptivity is given in Equation [5]. In this case, c refers specifically to the molar concentration of the analyte.

$$A = \epsilon bc \qquad [5]$$

where:

A, b as in Equation [4]

ϵ = molar absorptivity

c = concentration in units of molarity

Quantitative spectroscopy is dependent on the analyst being able to accurately measure the fraction of an incident light beam that is absorbed by the analyte in a given solution. This apparently simple task is somewhat complicated in actual practice due to processes other than analyte absorption that also result in significant decreases in the power of the incident beam. A pictorial summary of reflection and scattering processes that will decrease the power of an incident beam is given in Fig. 26-2. It is clear that these processes must be accounted for if a truly quantitative estimate of analyte absorption is necessary. In practice, a reference cell is used to correct for these processes. A reference cell is one that, in theory, exactly matches the sample absorption cell with the exception that it contains no analyte. A reference cell often is prepared by adding distilled water to an absorption cell. This reference cell is then placed in the path of the light beam, and the power of the radiation exiting the reference cell is measured and taken as P_0 for the sample cell. This procedure assumes that all processes except the selective absorption of radiation by the analyte are equivalent for the sample and reference cells. The absorbance actually measured in the laboratory approximates Equation [6].

$$A = \log (P_{solvent}/P_{analyte\ solution}) \cong \log (P_0/P) \qquad [6]$$
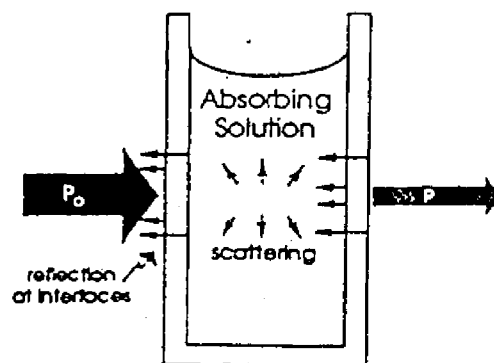
Factors contributing to the attenuation of a beam of radiation as it passes through a cuvette containing an absorbing solution.

where:

$$P = \text{radiant power of beam exiting cell} \atop \text{containing solvent (blank)}$$

$$P_{\text{analyte solution}} = \text{radiant power of beam exiting cell} \atop \text{containing analyte solution}$$

P_0, P as in Equation [1]

A as in Equation [3]

26.2.2 Deviations From Beer's Law

It should never be assumed that Beer's law is strictly obeyed. Indeed, there are several reasons for which the predicted linear relationship between absorbance and concentration may not be observed. In general, Beer's law is applicable only to dilute solutions, up to approximately 10 mM for most analytes. The actual concentration at which the law becomes limiting will depend on the chemistry of the analyte. As analyte concentrations increase, the intermolecular distances in a given sample solution will decrease, eventually reaching a point at which neighboring molecules mutually affect the charge distribution of the other. This perturbation may significantly affect the ability of the analyte to capture photons of a given wavelength; that is, it may alter the analyte's absorptivity (a). This causes the linear relationship between concentration and absorption to break down since the absorptivity term is the constant of proportionality in Beer's law (assuming a constant path length, b). Other chemical processes also may result in deviations from Beer's law, such as the reversible association–dissociation of analyte molecules or the ionization of a weak acid in an unbuffered solvent. In each of these cases, the predominant form of the analyte may change as the concentration is varied. If the different forms of the analyte, for example, ionized versus neutral, have different absorptivities (a), then a linear relationship between concentration and absorbance will not be observed.

A further source of deviation from Beer's law may arise from limitations in the instrumentation used for absorbance measurements. Beer's law strictly applies to situations in which the radiation passing through the sample is monochromatic, since under these conditions a single absorptivity value describes the interaction of the analyte with all the radiation passing through the sample. If the radiation passing through a sample is polychromatic and there is variability in the absorptivity constants for the different constituent wavelengths, then Beer's law will not be obeyed. An extreme example of this behavior occurs when radiation of the ideal wavelength and stray radiation of a wavelength that is not absorbed at all by the analyte simultaneously pass through the sample to the detector. In this case, the observed transmittance will be defined as in Equation [7]. Note that a limiting

absorbance value will be reached as $P_0 \gg P$, which will occur at relatively high concentrations of the analyte.

$$A = \log(P_0 + P_s)/(P + P_s) \qquad [7]$$

where:

P_s = radiant power of stray light

A as in Equation [3]

P, P_0 as in Equation [1]

26.2.3 Procedural Considerations

In general, the aim of quantitative measurements is to determine the concentration of an analyte with optimum precision and accuracy, in a minimal amount of time and at minimal cost. To accomplish this, it is essential that the analyst consider potential errors associated with each step in the methodology of a particular assay. Potential sources of error for spectroscopic assays include inappropriate sample preparation techniques, inappropriate controls, instrumental noise, and errors associated with inappropriate conditions for absorbance measurements (such as extreme absorbance/transmittance readings).

Sample preparation schemes for absorbance measurements vary considerably. In the simplest case, the analyte-containing solution may be measured directly following homogenization and clarification. Except for special cases, homogenization is required prior to any analysis to ensure a representative sample. Clarification of samples is essential prior to taking absorbance readings in order to avoid the apparent absorption due to scattering of light by turbid solutions. The reference solution for samples in this simplest case will be the sample solvent, the solvent being water or an aqueous buffer in many cases. In more complex situations, the analyte to be quantified may need to be chemically modified prior to making absorbance measurements. In these cases, the analyte that does not absorb radiation in an appropriate spectral range is specifically modified, resulting in a species with absorption characteristics compatible with a given spectrophotometric measurement. Specific reactions such as these are used in many colorimetric assays that are based on the absorption of radiation in the Vis range. The reference solution for these assays is prepared by treating the sample solvent in a manner identical with that of the sample. The reference solution therefore will help to correct for any absorbance due to the modifying reagents themselves and not the modified analyte.

A sample-holding cell or cuvette should be chosen after the general spectral region to be used in a spectrophotometric measurement has been determined. Sample holding cells vary in composition and dimensions. The sample holding cell should be composed of a material that does not absorb radiation in

the spectral region being used. Cells meeting this requirement for measurements in the UV range may be composed of quartz or fused silica. For the Vis range cells made of silicate glass are appropriate and inexpensive plastic cells also are available for some applications. The dimensions of the cell will be important with respect to the amount of solution required for a measurement and with regard to the path length term used in Beer's law. A typical absorption cell is 1 cm^2 and approximately 4.5 cm long. The path length for this traditional cell is 1 cm, and the minimum volume of solution needed for standard absorption measurements is approximately 1.5 ml. Absorption cells with path lengths ranging from 1 to 100 mm are commercially available. Narrow cells, approximately 4 mm in width, with optical path lengths of 1 cm, are also available. These narrow cells are convenient for absorbance measurements when limiting amounts of solution, less than 1 ml, are available.

In many cases, the analyst will need to choose an appropriate wavelength at which to make absorbance measurements. If possible, it is best to choose the wavelength at which the analyte demonstrates maximum absorbance and where the absorbance does not change rapidly with changes in wavelength (Fig. 26-3). This position usually corresponds to the apex of the highest absorption peak. Taking measurements at this apex has two advantages: (1) maximum sensitivity, defined as the absorbance change per unit change in analyte concentration and (2) greater adherence to Beer's law since the spectral region making up the
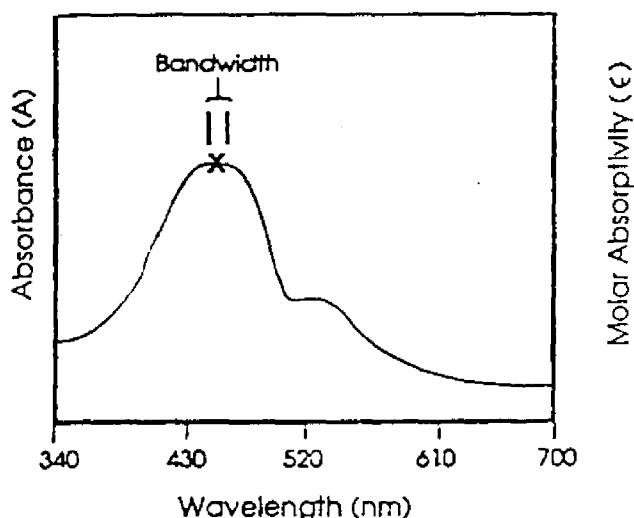
Hypothetical absorption spectrum between 340 and 700 nm. The effective bandwidth of the radiation used in obtaining the spectrum is assumed to be approximately 20 nm. Note that at the point indicated there is essentially no change in molar absorptivity over this wavelength range.

radiation beam is composed of wavelengths with relatively small differences in their molar absorptivities for the analyte being measured (Fig. 26-3). The latter point is important in that the radiation beam used in the analysis will be composed of a small continuous band of wavelengths centered about the wavelength indicated on the instrument's wavelength selector.

The actual absorbance measurement is made by first calibrating the instrument for 0% and then 100% transmittance. The 0% transmittance adjustment is made while the photodetector is screened from the incident radiation by means of an occluding shutter, mimicking infinite absorption. This adjustment sets the base level current or "dark current" to the appropriate level, such that the readout indicates zero. The 100% transmittance adjustment then is made with the occluding shutter open and an appropriate reference cell/solution in the light path. The reference cell itself should be equivalent to the cell that contains the sample, i.e., a "matched" set of cells is used. In many cases, the same cell is used for both the sample and reference solutions. The reference cell generally is filled with solvent, that often being distilled/deionized water for aqueous systems. The 100% T adjustment effectively sets T = 1 for the reference cell, which is equivalent to defining P_0 in Equation [1] as equivalent to the radiant power of the beam exiting the reference cell. The 0% T and 100% T settings should be confirmed as necessary throughout the assay. The sample cell that contains analyte then is measured without changing the adjustments. The adjustments made with the reference cell will effectively set the instrument to give a sample readout in terms of Equation [6]. The readout for the sample solution will be between 0 and 100% T. Most modern spectrophotometers allow the analyst to make readout measurements in either absorbance units or as percent transmittance. It is generally most convenient to make readings in absorbance units since, under optimum conditions, absorbance is directly proportional to concentration. When making measurements with an instrument that employs an analog swinging needle type of readout, it may be preferable to use the linear percent transmittance scale and then calculate the corresponding absorbance using Equation [3]. This is particularly true for measurements in which the percent transmittance is less than 20.

26.2.4 Calibration Curves

In most instances, it is advisable to use calibration curves for quantitative measurements. In food analysis, there are a large number of empirical assays for which calibration curves are essential. The calibration curve is used to establish the relationship between analyte concentration and absorbance. This relationship is established experimentally through the analysis of a

series of samples of known analyte concentration. The standard solutions are best prepared with the same reagents and at the same time as the unknown. The concentration range covered by the standard solutions must include that expected for the unknown. Typical calibration curves are depicted in Fig. 26-4. Linear calibration curves are expected for those systems that obey Beer's law. Nonlinear calibration curves are used for some assays, but linear relationships generally are preferred due to the ease of processing the data. Nonlinear calibration curves may be due to concentration-dependent changes in the chemistry of the system or to limitations inherent in the instruments used for the assay. The nonlinear calibration curve in Fig. 26-4b reflects the fact that the calibration sensitivity, defined as change in absorbance per unit change in analyte concentration, is not constant. For the case depicted in Fig. 26-4b, the assay's concentration-dependent decrease in sensitivity obviously begins to limit its usefulness at analyte concentrations above 10 mM.

In many cases, truly representative calibration standards cannot be prepared due to the complexity of the unknown sample. This scenario must be assumed when insufficient information is available on the extent of interfering compounds in the unknown. Interfering compounds include those that absorb radiation in the same spectral region as the analyte, those that influ-

ence the absorbance of the analyte, and those compounds that react with modifying reagents that are supposedly specific for the analyte. This means that calibration curves are potentially in error if the unknown and the standards differ with respect to pH, ionic strength, viscosity, types of impurities, and the like. In these cases, it is advisable to calibrate the assay system by using a standard addition protocol. One such protocol goes as follows: To a series of flasks add a constant volume of the unknown (V_u) for which you are trying to determine the analyte concentration (C_u). Next, to each individual flask add a known volume (V_s) of a standard analyte solution of concentration C_s, such that each flask receives a unique volume of standard. The resulting series of flasks will contain identical volumes of the unknown and different volumes of the standard solution. Next, dilute all flasks to the same total volume, V_t. Each of the flasks then is assayed, with each flask treated identically. If Beer's law is obeyed, then the measured absorbance of each flask will be proportional to the total analyte concentration as defined in Equation [8].

$$A = k \left[(V_s C_s + V_u C_u)/(V_t) \right] \qquad [8]$$

where:

V_s = volume of standard
V_u = volume of unknown
V_t = total volume
C_s = concentration of standard
C_u = concentration of unknown
k = proportionality constant (path length × absorptivity)

The results from the assays are then plotted with the volume of standard added to each flask (V_s) as the independent variable and the resulting absorbance (A) as the dependent variable (Fig. 26-5). Assuming Beer's law, the line describing the relationship will be as in

(a)

(b)

Analyte Concentration (mM)
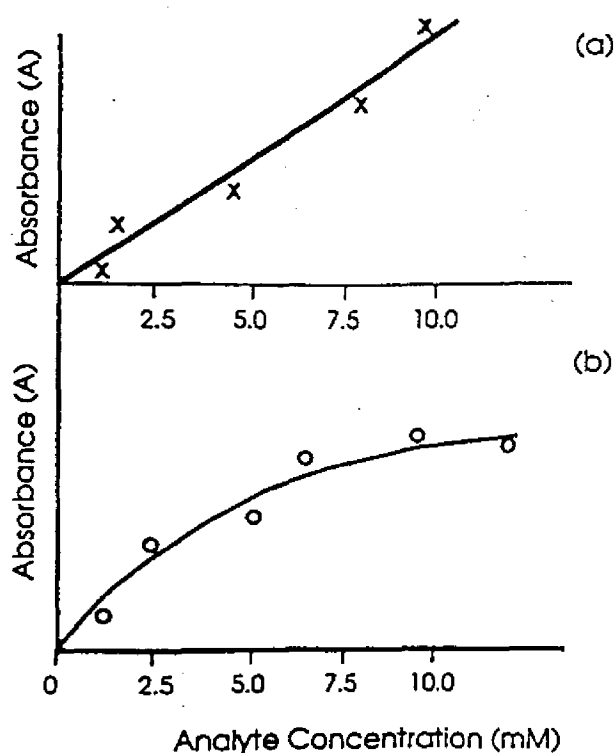
26-4 figure

Linear (a) and nonlinear (b) calibration curves typically encountered in quantitative absorption spectroscopy.

Volume of standard $(V_s$,mls)
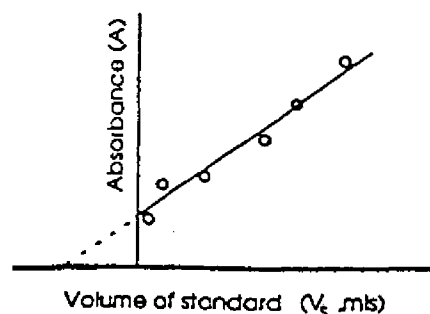
26-5 figure

Calibration curve for the determination of the analyte concentration in an unknown using a standard addition protocol. A, absorbance; V_s, volume of standard analyte solution; as discussed in text.

Equation [9], in which all terms other than V_s and A are constants. Taking the ratio of the slope of the plotted line (Equation [10]) to the line's intercept (Equation [11]) and rearranging gives Equation [12], from which the concentration of the unknown, C_u, can be calculated since C_s and V_u are experimentally defined constants.

$$A = kC_sV_s/V_T + V_uC_uk/V_T \qquad [9]$$

$$\text{slope} = kC_s/V_T \qquad [10]$$

$$\text{intercept} = V_uC_uk/V_T \qquad [11]$$

$$C_u =$$
$$(\text{measured intercept/measured slope})(C_s/V_u) \qquad [12]$$

where:

V_s, V_u, V_T, C_s, C_u, and K as in Equation [8]

26.2.5 Effect of Indiscriminate Instrumental Error on the Precision of Absorption Measurements

All spectrophotometric assays will have some level of indiscriminant error associated with the absorbance/transmittance measurement itself. Indiscriminant error of this type often is referred to as instrument noise. It is important that the assay be designed such that this source of error is minimized, the objective being to keep this source of error low relative to the variability associated with other aspects of the assay, such as sample preparation, subsampling, reagent handling, and so on. Indiscriminant instrumental error is observed with repeated measurements of a single homogeneous sample. The relative concentration uncertainty resulting from this error is not constant over the entire percent transmittance range (0–100%). Measurements at intermediate transmittance values tend to have lower relative errors, thus greater relative precision, than measurements made at either very high or very low transmittance. Relative concentration uncertainty or relative error may be defined as S_c/C, where S_c = sample standard deviation and C = measured concentration. Relative concentration uncertainties of from 0.5% to 1.5% are to be expected for absorbance/transmittance measurements taken in the optimal range. The optimal range for absorbance measurements on simple, less expensive spectrophotometers is from approximately 0.2 to 0.8 absorbance units, or from 15% to 65% transmittance. On more sophisticated instruments, the range for optimum absorbance readings may be extended up to 1.5 or greater. To be safe, it is prudent to always make absorbance readings under conditions at which the absorbance of the analyte solution is less than 1.0. If there is an anticipated need to make measurements at absorbance readings greater than 1.0,

then the relative precision of the spectrophotometer should be established experimentally by repetitive measurements of appropriate samples. Absorbance readings outside the optimal range of the instrument may be used, but the analyst must be prepared to account for the higher relative error associated with these extreme readings. When absorbance readings approach the limits of the instrumentation, then relatively large differences in analyte concentrations may not be detected.

26.2.6 Instrumentation

There are many variations of spectrophotometers available for UV-Vis spectrophotometry. Some instruments are designed for operation in only the visible range, while others encompass both the UV and Vis range. Instruments may differ with respect to design, quality of components, and versatility. A basic spectrophotometer is composed of five essential components: the light source, the monochromator, the sample/reference holder, the radiation detector, and a readout device. A power supply is required for instrument operation. A schematic depicting component interrelationships is shown in Fig. 26-6.

Light sources used in spectrophotometers must continuously emit a strong band of radiation encompassing the entire wavelength range for which the instrument is designed. The power of the emitted radiation must be sufficient for adequate detector response, and it should not vary sharply with changes in wavelength or drift significantly over the experimental time scale. The most common radiation source for Vis spectrophotometers is the tungsten filament lamp. These lamps emit adequate radiation covering the wavelength region from 350 to 2500 nm. Consequently, tungsten filament lamps also are employed in near-infrared spectroscopy. The most common radiation sources for measurements in the UV range are deuterium electrical-discharge lamps. These sources provide a continuous radiation spectrum from approximately 160 nm through 375 nm. These lamps employ quartz windows and should be used in conjunction with quartz sample holders, since glass significantly absorbs radiation below 350 nm.

The component that functions to isolate the specific, narrow, continuous group of wavelengths to be used in the spectroscopic assay is the monochromator. The monochromator is so named because light of a single wavelength is termed monochromatic. Theoretically, polychromatic radiation from the source enters the monochromator, is dispersed according to wavelength, and monochromatic radiation of a selected wavelength exits the monochromator. In practice, light exiting the monochromator is not of a single wavelength, but rather it consists of a narrow continuous
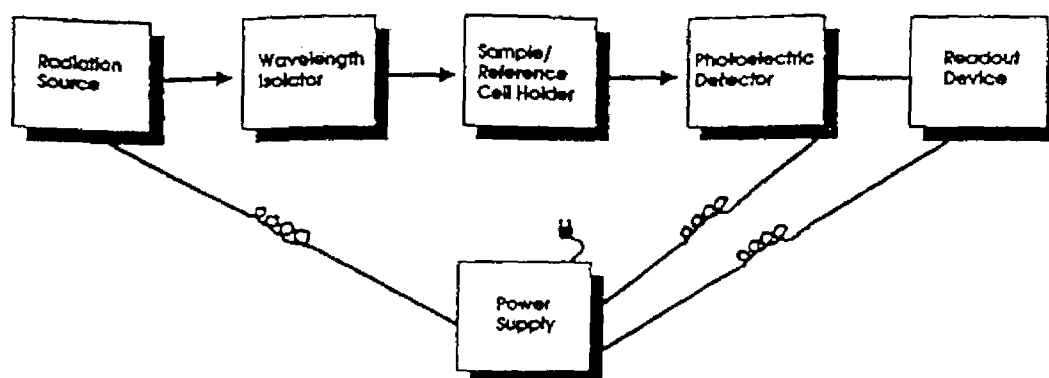
26-6 **figure** Arrangement of components in a simple single-beam, UV-Vis absorption spectrophotometer.
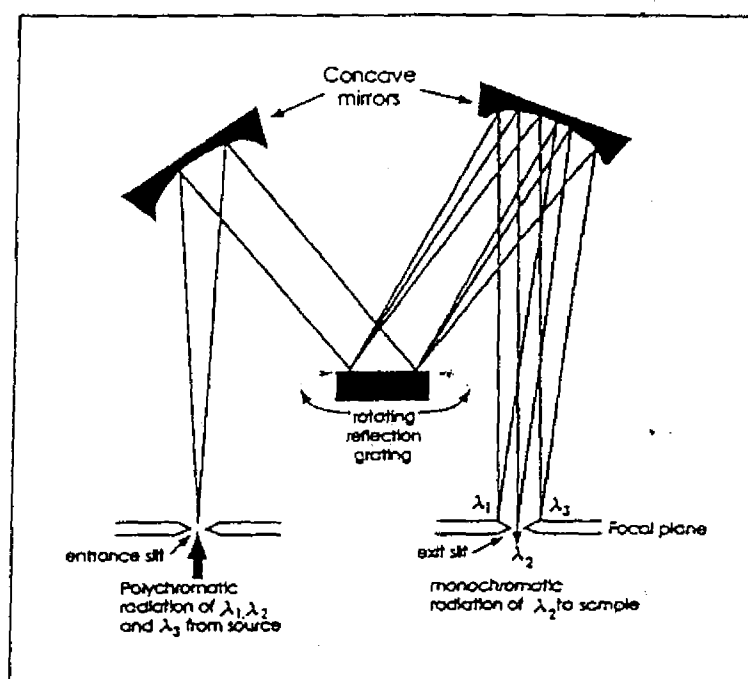
26-7 **figure** Schematic of a monochromator employing a reflection grating as the dispersing element. The concave mirrors serve to collimate the radiation into a beam of parallel rays.

band of wavelengths. A representative monochromator is depicted in Fig. 26-7. As illustrated, a typical monochromator is composed of entrance and exit slits, concave mirror(s), and a dispersing element (the grating in this particular example). Polychromatic light enters the monochromator through the entrance slit and is then culminated by a concave mirror. The culminated polychromatic radiation is then dispersed, dispersion being the physical separation in space of radiation of different wavelengths. The radiation of

different wavelengths is then reflected from a concave mirror that focuses the different wavelengths of light sequentially along the focal plane. The radiation that aligns with the exit slit in the focal plane thus is emitted from the monochromator. The radiation emanating from the monochromator will consist of a narrow range of wavelengths presumably centered around the wavelength specified on the wavelength selection control of the instrument.

The size of the wavelength range passing out of

the exit slit of the monochromator is termed the bandwidth of the emitted radiation. Many spectrophotometers allow the analyst to adjust the size of the monochromator exit slit (and entrance slit) and, consequently, the bandwidth of the emitted radiation. Decreasing the exit slit width will decrease the associated bandwidth and the radiant power of the emitted beam. Conversely, further opening of the exit slit will result in a beam of greater radiant power, but one that has a larger bandwidth. In some cases where resolution is critical, such as some qualitative work, the narrower slit width may be advised. However, in most quantitative work a relatively open slit may be used since adsorption peaks in the UV-Vis range generally are broad relative to spectral bandwidths. Also, the signal-to-noise ratio associated with transmittance measurements is improved due to the higher radiant power of the measured beam.

The effective bandwidth of a monochromator is determined not only by the slit width but also by the quality of its dispersing element. The dispersing element functions to spread out the radiation according to wavelength. Reflection gratings, as depicted in Fig. 26-8, are the most commonly used dispersing elements in modern spectrophotometers. Gratings sometimes are referred to as diffraction gratings because the separation of component wavelengths is dependent on the different wavelengths being diffracted at different angles relative to the grating normal. A reflection grating incorporates a reflective surface in which a series of closely spaced grooves has been etched, typically between 1200 and 1400 grooves per millimeter. The grooves themselves serve to break up the reflective surface such that each point of reflection behaves as an independent point source of radiation.

Referring to Fig. 26-8, lines 1 and 2 represent rays of parallel monochromatic radiation that are in phase
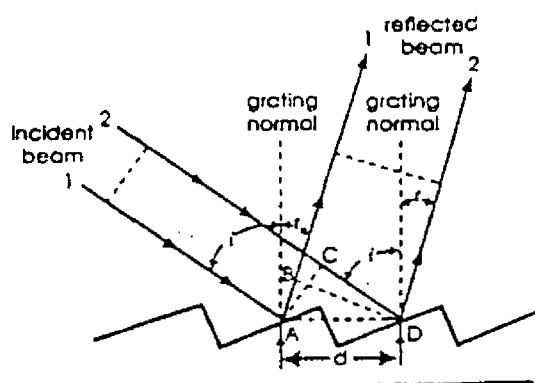
and that strike the grating surface at an angle i to the normal. Maximum constructive interference of this radiation is depicted as occurring at an angle r to the normal. At all other angles, the two rays will partially or completely cancel each other. Radiation of a different wavelength would show maximum constructive interference at a different angle to the normal. The wavelength dependence of the diffraction angle can be rationalized by considering the relative distance the photons of rays 1 and 2 travel and assuming that maximum constructive interference occurs when the waves associated with the photons are completely in phase. Referring to Fig. 26-8, prior to reflection, photon 2 travels a distance CD greater than photon 1. After reflection, photon 1 travels a distance AB greater than photon 2. Hence, the waves associated with photons 1 and 2 will remain in phase after reflection only if the net difference in the distance traveled is an integral multiple of their wavelength. Note that for a different angle r the distance AB would change and, consequently, the net distance $CD - AB$ would be an integral multiple of a different wavelength. The net result is that the component wavelengths are each diffracted at their own unique angles r.

In a spectroscopic measurement, the light transmitted through the reference or sample cell is quantified by a detector. The detector is designed to produce an electric signal when it is struck by photons. An ideal detector would give a signal directly proportional to the radiant power of the beam striking it; it would have a high signal-to-noise ratio; and it would have a relatively constant response to light of different wavelengths, such that it was applicable to a wide range of the radiation spectrum. There are several types and designs of radiation detectors currently in use. Two of the more popular detectors used in modern spectrophotometers are the phototube and the photomultiplier tube. Both detectors function by converting the energy associated with incoming photons into electrical current. The phototube consists of a semicylindrical cathode covered with a photoemissive surface and a wire anode, the electrodes being housed under vacuum in a transparent tube (Fig. 26-9a). When photons strike the photoemissive surface of the cathode, there is an emission of electrons, and the freed electrons then are collected at the anode. The net result of this process is that a measurable current is created. The number of electrons emitted from the cathode and the subsequent current through the system are directly proportional to the number of photons, or radiant power of the beam, impinging on the photoemissive surface. The photomultiplier tube is of similar design. However, in the photomultiplier tube there is an amplification of the number of electrons collected at the anode per photon striking the photoemissive sur-

26-8
figure

Schematic illustrating the property of diffraction from a reflection grating. Each reflected point source of radiation is separated by a distance d.

(a)

(b)

Photomultiplier Arrangement
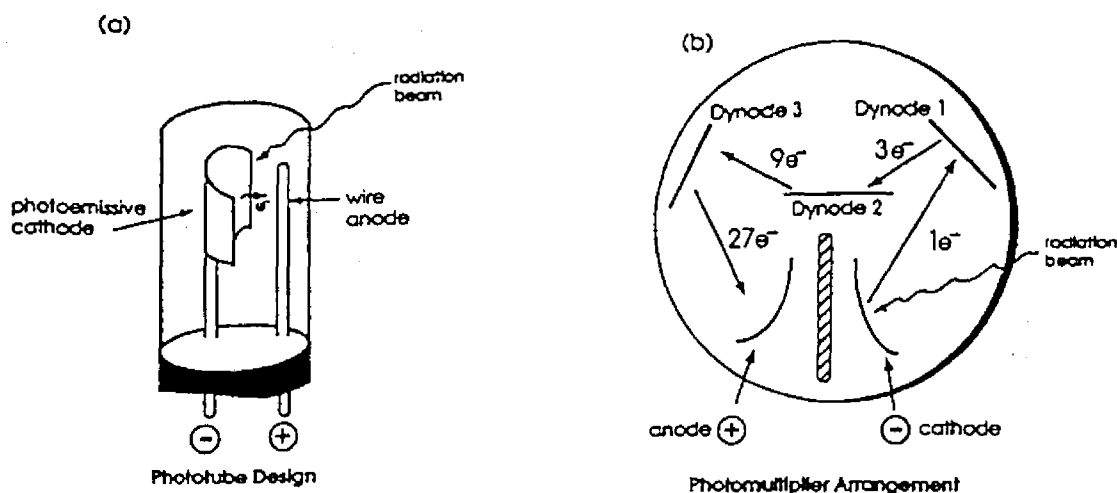
26-9 figure   Schematic diagram of a typical phototube design (a) and the cathode–dynode–anode arrangement of a representative photomultiplier tube (b).

face of the cathode (Fig. 26-9b). The electrons originally emitted from the cathode surface are attracted to a dynode with a relative positive charge. At the dynode, the electrons strike the surface, causing the emission of several more electrons per original electron, resulting in an amplification of the signal. Signal amplification continues in this manner, as photomultiplier tubes generally contain a series of such dynodes, with electron amplification occurring at each dynode. The cascade continues until the electrons emitted from the final dynode are collected at the anode of the photomultiplier tube. The final gain may be as many as 10^6–10^9 electrons collected per photon.

The signal from the detector generally is amplified and then displayed in a usable form to the analyst. The final form in which the signal is displayed will depend on the complexity of the system. In the simplest case, the analog signal from the detector is displayed on an analog meter through the position of a needle on a meter face calibrated in percent transmission or absorbance. Analog readouts are adequate for most routine analytical purposes; however, analog meters are somewhat more difficult to read and, hence, the resulting data are expected to have somewhat lower precision than that obtained on a digital readout (assuming the digital readout is given to enough places). Digital readouts express the signal as numbers on the face of a meter. In these cases, there is an obvious requirement for signal processing between the analog output of the detector and the final digital display. In virtually all cases, the signal processor is capable of presenting the final readout in terms of either absorbance or transmittance. Many of the newer instruments include microprocessors capable of more extensive data manipula-

tions on the digitized signal. For example, the readouts of some spectrophotometers may be in concentration units, provided the instrument has been correctly calibrated with appropriate reference standards.

26.2.7 Instrument Design

The optical systems of spectrophotometers fall into one of two general categories: They are either single-beam or double-beam instruments. In a single-beam instrument, the radiant beam follows only one path, that going from the source through the sample to the detector (Fig. 26-6). When using a single-beam instrument, the analyst generally measures the transmittance of a sample after first establishing 100% T, or P_0 with a reference sample or blank. The blank and the sample are read sequentially since there is but a single light path going through a single cell-holding compartment. In a double-beam instrument, the beam is split such that one half of the beam goes through one cell-holding compartment and the other half of the beam passes through a second. The schematic of Fig. 26-10 illustrates a double-beam optical system in which the beam is split in time between the sample and reference cell. In this design, the beam is alternately passed through the sample and reference cells by means of a rotating sector mirror with alternating reflective and transparent sectors. The double-beam design allows the analyst to simultaneously measure and compare the relative absorbance of a sample and a reference cell. The advantage of this design is that it will compensate for deviations or drifts in the radiant output of the source since the sample and reference cells are compared many times per second. The disadvantage of the double-beam design is that the
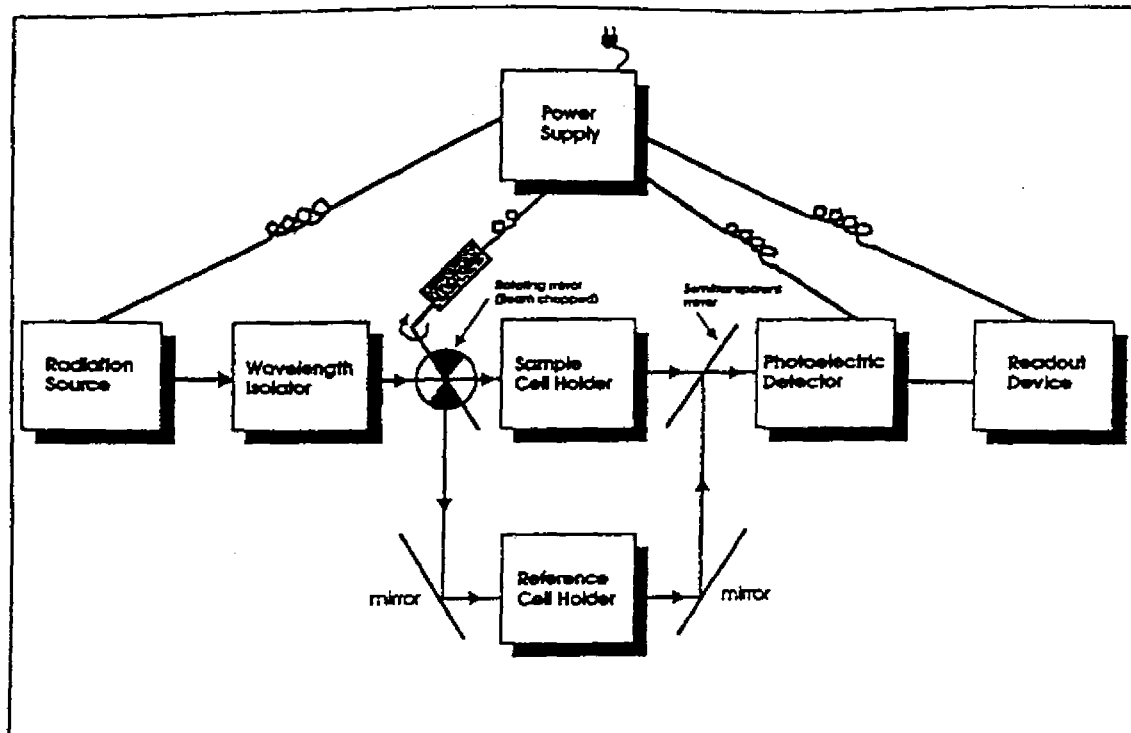
Arrangement of components in a representative double-beam UV-Vis absorption spectrophotometer. The incident beam is alternatively passed through the sample and reference cells by means of a rotating beam chopper.

radiant power of the incident beam is diminished because the beam is split. The lower energy throughput of the double-beam design is generally associated with inferior signal-to-noise ratios. Computerized single-beam spectrophotometers now are available that claim to have the benefits of both the single- and double-beam designs. Their manufacturers report that previously troublesome source and detector drift and noise problems have been stabilized such that simultaneous reading of the reference and sample cell is not necessary. With these instruments, the reference and sample cells are read sequentially, and the data are stored, then processed, by the associated computer.

The Spectronic® 20 is a classic example of a simple single-beam visible spectrophotometer (Fig. 26-11). The white light emitted from the source passes into the monochromator via its entrance slit; the light is then dispersed into a spectrum by a diffraction grating; and a portion of the resulting spectrum then leaves the monochromator via the exit slit. The radiation emitted from the monochromator passes through a sample compartment and strikes the measuring phototube, resulting in an induced photocurrent that is proportional to the intensity of impinging light. The lenses depicted in Fig. 26-11 function in series to focus the light image on the focal plane that contains the exit slit.

To change the portion of the spectrum exiting the monochromator, one rotates the reflecting grating by means of the wavelength cam. A shutter automatically blocks light from exiting the monochromator when no sample/reference cell is in the instrument; the zero percent T adjustment is made under these conditions. The light control occluder is used to adjust the radiant power of the beam exiting the monochromator. The occluder consists of an opaque strip with a V-shaped opening that can be physically moved in or out of the beam path. The occluder is used to make the 100% T adjustment when an appropriate reference cell is in the instrument.

26.3 FLUORESCENCE SPECTROSCOPY

The technique of fluorescence spectroscopy is generally one to three orders of magnitude more sensitive than corresponding absorption spectroscopy methods. In fluorescence spectroscopy, the signal being measured is the electromagnetic radiation that is emitted from the analyte as it relaxes from an excited electronic energy level to its corresponding ground state. The analyte is originally activated to the higher energy level by the absorption of radiation in the UV or Vis
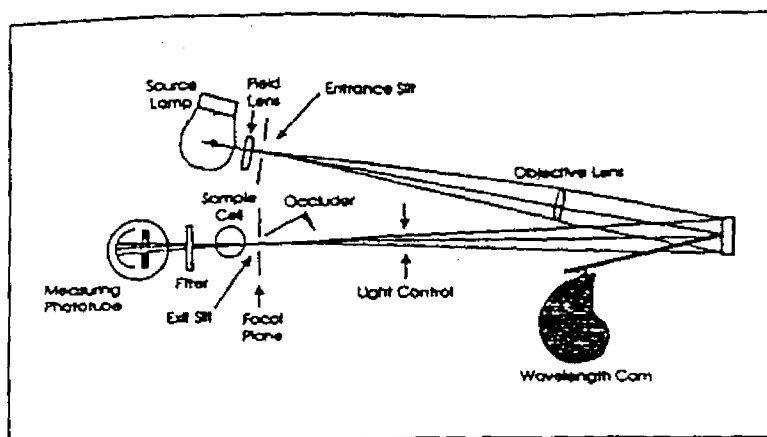
Optical system for the SPECTRONIC® 20 spectrophotometer. (Figure 3, *Bausch & Lomb SPECTRONIC® 20* Spectrophotometer Operator's Manual. Courtesy of Milton Roy Company, Rochester, NY, a subsidiary of Sundstrand Corporation.)

range. The processes of activation and deactivation occur simultaneously during a fluorescence measurement. For each unique molecular system, there will be an optimum radiation wavelength for sample excitation and another, of longer wavelength, for monitoring fluorescence emission. The respective wavelengths for excitation and emission will depend on the chemistry of the system under study.

The instrumentation used in fluorescence spectroscopy is composed of essentially the same components as the corresponding instrumentation used in UV-Vis absorption spectroscopy. However, there are definite differences in the arrangement of the optical systems used for the two types of spectroscopy (compare Figs. 26-6 and 26-12). In fluorometers and spectrofluorometers, there is a need for two wavelength selectors, one for the excitation beam and one for the emission beam. In some simple fluorometers, both wavelength selectors are filters such that the excitation and emission wavelengths are fixed. In more sophisticated spectrofluorometers, the excitation and emission wavelengths are selected by means of grating monochromators. The photon detector of fluorescence instrumentation is generally arranged such that the emitted radiation that strikes the detector is traveling at an angle of 90°C relative to the axis of the excitation beam. This detector placement minimizes signal interference due to transmitted source radiation and radiation scattered from the sample.

The radiant power of the fluorescence beam (P_F) emitted from a fluorescent sample is proportional to the change in the radiant power of the source beam as it passes through the sample cell (Equation [13]). Expressing this another way, the radiant power of the fluorescence beam will be proportional to the number of photons absorbed by the sample.
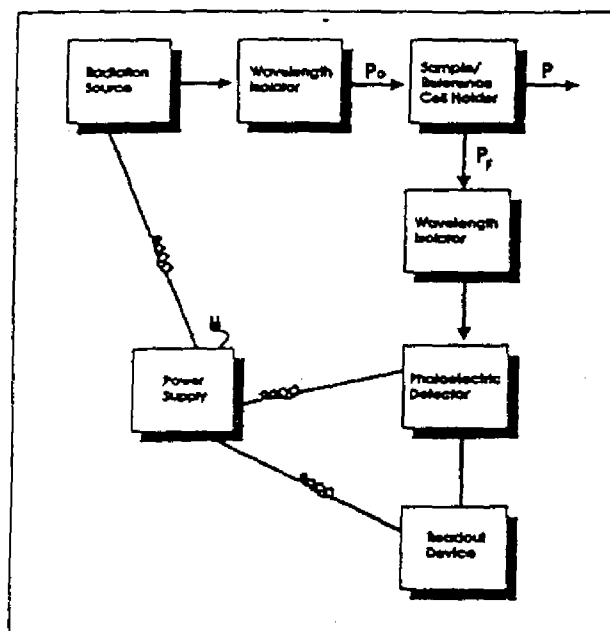
Schematic diagram depicting the arrangement of the source, excitation and emission wavelength selectors, sample cell, photoelectric detector, and readout device for a representative fluorometer or spectrofluorometer.

$$P_F = \varphi(P_0 - P) \qquad [13]$$

where:

P_F = radiant power of beam emitted from fluorescent cell

φ = constant of proportionality

P_0, P as in Equation [1]

The constant of proportionality used in Equation

[13] is termed the **quantum efficiency** (φ), which is specific for any given system. The quantum efficiency equals the ratio of the total number of photons emitted to the total number of photons absorbed. Combining Equations [3] and [5] allows one to define P in terms of the analyte concentration and P_0, as given in Equation [14].

$$P = P_0 10^{-\epsilon bc}$$ [14]

where:

P_0, P as in Equation [1]
ϵ, b, c as in Equation [5]

Substitution of Equation [14] into Equation [13] gives an expression that relates the radiant power of the fluorescent beam to the analyte concentration and P_0, as shown in Equation [15]. At low analyte concentration, $\epsilon bc < 0.01$, Equation [15] may be reduced to the expression of Equation [16]. Further grouping of terms leads to the expression of Equation [17], where k incorporates all terms other than P_0 and c.

$$P_F = \varphi P_0(1 - 10^{-\epsilon bc})$$ [15]

$$P_F = \varphi P_0 2.303 \, \epsilon bc$$ [16]

$$P_F = k P_0 c$$ [17]

where:

k = constant of proportionality
P_0 as in Equation [13]
c as in Equation [5]

Equation [17] is particularly useful because it emphasizes two important points that are valid for the conditions assumed when deriving the equation, particularly the assumption that analyte concentrations are kept relatively low. First, the fluorescent signal will be directly proportional to the analyte concentration, assuming other parameters are kept constant. This is very useful because a linear relationship between signal and analyte concentration simplifies data processing and assay troubleshooting. Second, the sensitivity of a fluorescent assay is proportional to P_0, the power of the incident beam, the implication being that the sensitivity of a fluorescent assay may be modified by adjusting the source output.

Equations [16] and [17] will eventually break down if analyte concentrations are increased to relatively high values. Therefore, the linear concentration range for each assay should be determined experimentally. A representative calibration curve for a fluorescence assay is presented in Fig. 26-13. The nonlinear portion of the curve at relatively high analyte concentrations results from decreases in the fluorescence yield per
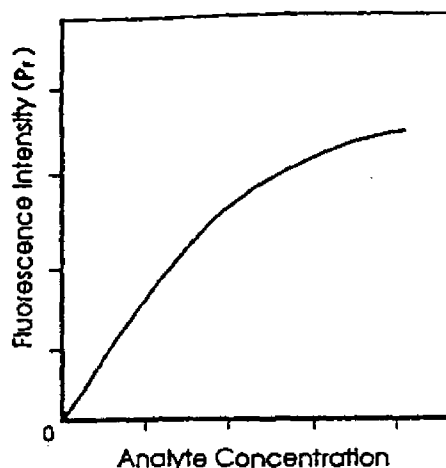
Relationship between the solution concentration of a fluorescent analyte and that solution's fluorescence intensity. Note that there is a linear relationship at relatively low analyte concentrations that eventually goes nonlinear as the analyte concentration increases.

unit concentration. The fluorescence yield for any given sample also is dependent on its environment. Temperature, solvent, impurities, and pH may influence this parameter. Consequently, it is imperative that these environmental parameters be accounted for in the experimental design of fluorescence assays. This may be particularly important in the preparation of appropriate reference standards for quantitative work.

26.4 SUMMARY

UV and Vis absorption and fluorescence spectroscop are used widely in food analysis. These technique may be used for either qualitative or quantitative mer surements. Qualitative measurements are based on th premise that each analyte has a unique set of energy spacings that will dictate its absorption/emission spectrum. Hence, qualitative assays generally are based the analysis of the absorption or emission spectrum the analyte. In contrast, quantitative assays most oft are based on measuring the absorbance or fluorescer of the analyte solution at one wavelength. Quantitat absorption assays are based on the premise that absorbance of the test solution will be a function of solution's analyte concentration.

Under optimum conditions, there is a direct li relationship between a solution's absorbance and analyte concentration. The equation describing thi ear relationship is known as Beer's law. The appl bility of Beer's law to any given assay always sh be verified experimentally by means of a calibr curve. The calibration curve should be establish the same time and under the same conditions th

used to measure the test solution. The analyte concentration of the test solution then should be estimated from the established calibration curve.

Molecular fluorescence methods are based on the measurement of radiation emitted from excited analyte molecules as they relax to lower energy levels. The analytes are raised to the excited state as a result of photon absorption. The processes of photon absorption and fluorescence emission occur simultaneously during the assay. Quantitative fluorescence assays are generally one to three orders of magnitude more sensitive than corresponding absorption assays. Like absorption assays, under optimal conditions there will be a direct linear relationship between the fluorescence intensity and the concentration of the analyte in the unknown solution. Most molecules do not fluoresce and, hence, cannot be assayed by fluorescence methods.

The instrumentation used for absorption and fluorescence methods have similar components, including a radiation source, wavelength selector(s), sample holding cell(s), radiation detector(s), and a readout device.

26.5 STUDY QUESTIONS

1. Why is it common to use absorbance values rather than transmittance values when doing quantitative UV-Vis spectroscopy?
2. For a particular assay, your plot of absorbance versus concentration is not linear. Explain the possible reasons for this.
3. What criteria should be used to choose an appropriate wavelength at which to make absorbance measurements, and why is that choice so important?
4. In a particular assay, the absorbance reading on the spectrophotometer for one sample is 2.033 and for another sample 0.032. Would you trust these values? Why or why not?
5. Explain the difference between electromagnetic radiation in the UV and Vis ranges. How does quantitative spectroscopy using the UV range differ from that using the Vis range?
6. What is actually happening inside the spectrophotometer when the analyst "sets" the wavelength for a particular assay?
7. Considering a typical spectrophotometer, what is the effect of decreasing the exit slit width of the monochromator on the light incident to the sample?
8. Describe the similarities and differences between a phototube and a photomultiplier tube. What is the advantage of one over the other?
9. Your lab has been using an old single-beam spectrophotometer that must now be replaced by a new spectrophotometer. You obtain sales literature that describes single-beam and double-beam instruments. What are the basic differences between a single-beam and a double-beam spectrophotometer, and what are the advantages and disadvantages of each?

10. Explain the similarities and differences between UV-Vis spectroscopy and fluorescence spectroscopy with regard to instrumentation and principles involved. What is the advantage of using fluorescence spectroscopy?

26.6 PRACTICE PROBLEMS

1. A particular food coloring has a molar absorptivity of 3.3×10^3 cm^{-1} M^{-1} at 510 nm.
   a. What will be the absorbance of a $2 \times 10^{-4}M$ solution in a 1 cm cuvette at 510 nm?
   b. What will be the percent transmittance of the solution in (a)?
2. a. You measure the percent transmittance of a solution containing chromophore X at 400 nm in a 1 cm pathlength cuvette and find it to be 50%. What is the absorbance of this solution?
   b. What is the molar absorptivity of chromophore X if the concentration of X in the solution measured in question 2a is 0.5 mM?
   c. What is the concentration range of chromophore X that can be assayed if, when using a sample cell of pathlength 1, you are required to keep the absorbance between 0.2 and 0.8?
3. What is the concentration of compound Y in an unknown solution if the solution has an absorbance of 0.846 in a glass cuvette with a pathlength of 0.2 cm? The absorptivity of compound Y is 54.2 cm^{-1} $(mg/ml)^{-1}$ under the conditions used for the absorption measurement.
4. a. What is the molar absorptivity of compound Z at 295 nm and 348 nm, given the absorption spectrum shown in Fig. 26-14 (which was obtained using a UV-Vis spectrophotometer and a 1 mM solution of compound Z in a sample cell with a pathlength of 1 cm)?
   b. Now you have decided to make quantitative measurements of the level of compound Z in different
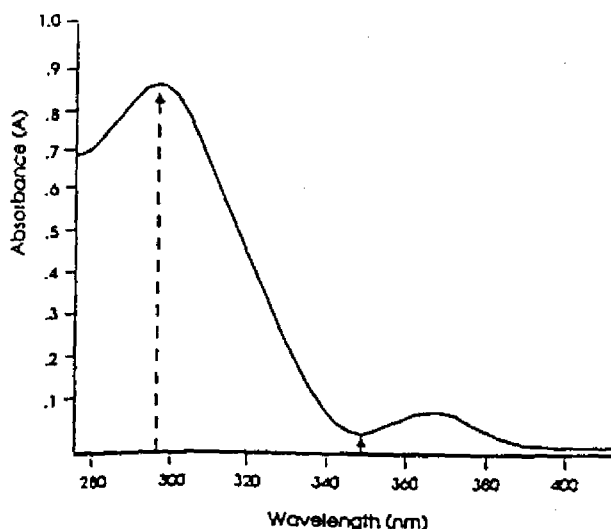
Absorption spectrum of compound Z, to be used in conjunction with problems 4a and 4b.

solutions. Based on the above spectrum, which wavelength will you use for your measurements? Give two reasons why this is the optimum wavelength.

Answers

1. $a = 0.76$, $b = 17.4$; 2. $a = 301$, $b = 602$ cm^{-1} M^{-1}, $c = 0.33 \times 10^{-3}M$ to $1.33 \times 10^{-3}M$; 3.0.078 mg/ml; 4. $a = 860$ at 295 nm, 60 at 348 nm; $b = 295$ nm; optimum sensitivity and more likely to adhere to Beer's law.

26.7 RESOURCE MATERIALS

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Infrared Spectroscopy

Randy L. Wehling

27.1 INTRODUCTION

Infrared (IR) spectroscopy refers to measurement of the absorption of different frequencies of infrared radiation by foods or other solids, liquids, or gases. IR spectroscopy began in 1800 with an experiment by Herschel. When he used a prism to create a spectrum from white light and placed a thermometer at a point just beyond the red region of the spectrum, he noted an increase in temperature. This was the first observation of the effects of IR radiation. By the 1940s, IR spectroscopy had become an important tool used by chemists to identify functional groups in organic compounds. In the 1970s, commercial near IR reflectance instruments were introduced that provided rapid quantitative determinations of moisture, protein, and fat in cereal grains and other foods. Today, IR spectroscopy is used widely in the food industry for both qualitative and quantitative analysis of ingredients and finished foods.

In this chapter, the techniques of mid- and near-IR spectroscopy are described, including the principles by which molecules absorb IR radiation, the components and configuration of commercial IR spectrometers, sampling methods for IR spectroscopy, and qualitative and quantitative applications of these techniques to food analysis.

27.2 PRINCIPLES OF INFRARED (IR) SPECTROSCOPY

27.2.1 The IR Region of the Electromagnetic Spectrum

Infrared radiation is electromagnetic energy with wavelengths (λ) longer than visible light but shorter than microwaves. Generally, wavelengths from 0.8 to 100 micrometers (μm) can be used for IR spectroscopy and are divided into the near-IR (0.8–2.5 μm), the mid-IR (2.5–15 μm), and the far IR (15–100 μm) regions. One μm is equal to 1×10^{-6} m. The near- and mid-IR regions of the spectrum are most useful for quantitative and qualitative analysis of foods.

IR radiation also can be measured in terms of its frequency, which is useful because frequency is directly related to the energy of the radiation by the following relationship:

$$E = h\nu \qquad [1]$$

where:

E = the energy of the system
h = Planck's constant
v = the frequency in hertz

Frequencies are also commonly expressed as wave-

numbers ($\bar{\nu}$, in reciprocal centimeters, cm^{-1}). Wavenumbers are calculated as follows:

$$\bar{\nu} = 1/(\lambda \text{ in cm}) = 10^4/(\lambda \text{ in } \mu m) \qquad [2]$$

27.2.2 Molecular Vibrations

A molecule can absorb IR radiation if it vibrates in such a way that its charge distribution, and therefore its electric dipole moment, changes during the vibration. Although there are many possible vibrations in a polyatomic molecule, the most important vibrations that produce a change in dipole moment are stretching and bending (scissoring) motions. Examples of these vibrations for the water molecule are shown in Fig. 27-1. Note that the stretching motions vibrate at a higher frequency than the scissoring motion, indicating that more energy is required.

27.2.3 Factors Affecting the Frequency of Vibration

The energy level for any molecular vibration is given by the following equation:

$$E = (v + 1/2)(h/2\pi) \sqrt{k \left/ \frac{m_1 m_2}{m_1 + m_2} \right.} \qquad [3]$$

where:

v = the vibrational quantum number (positive integer values, including zero, only)
h = Planck's constant
k = the force constant of the bond
m_1 and m_2 = the masses of the individual atoms involved in the vibration

Note that the vibrational energy, and therefore the frequency of vibration, is directly proportional to the strength of the bond and inversely proportional to the mass of the molecular system. The vibrating molecular functional group can absorb radiant energy to move from the lowest ($v = 0$) vibrational state to the first excited ($v = 1$) state, and the frequency of radiation that will make this occur is identical to the initial frequency of vibration of the bond. This frequency is referred to as the fundamental absorption. Molecules also can absorb radiation to move to a higher ($v = 2$ or 3) excited state, such that the frequency of the radiation absorbed is two or three times that of the fundamental frequency. These absorptions are referred to as overtones, and the intensity of these absorptions is much lower than the fundamental since these transitions are less favored. The overall result is that each functional group within the molecule absorbs IR radiation in distinct wavelength bands rather than as a continuum.

SYMMETRICAL STRETCH

ASYMMETRIC STRETCH
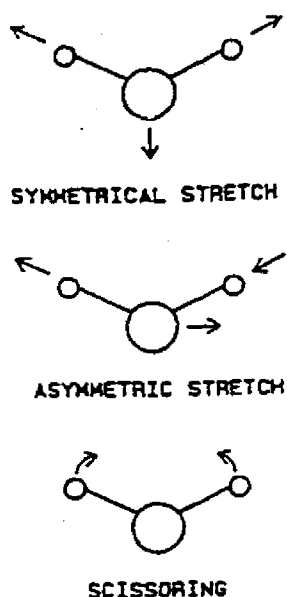
SCISSORING

27-1 figure Vibrational modes of the water molecule. Frequencies of the fundamental vibration for the symmetrical stretch, asymmetric stretch, and scissoring motion are 3652 cm^{-1}, 3756 cm^{-1} and 1596 cm^{-1}, respectively.

27.3 MID-IR SPECTROSCOPY

Mid-IR spectroscopy measures a sample's ability to absorb light in the 2.5–15 μm (4000–650 cm^{-1}) region. Fundamental absorptions are primarily observed in this spectral region.

27.3.1 Instrumentation

Two types of spectrometers are routinely used for mid-IR spectroscopy: dispersive instruments and Fourier transform instruments. Most newer instruments are of the Fourier transform type.

27.3.1.1 Dispersive Instruments

Dispersive instruments use a monochromator to disperse the individual frequencies of radiation and sequentially pass them through the sample so that the absorption of each frequency can be measured. IR spectrometers have components similar to ultraviolet-visible (UV-Vis) spectrometers, including a radiation source, a monochromator, a sample holder, and a detector connected to an amplifier system for recording the spectra. Most IR spectrometers are double-beam instruments.

A common IR source is a coil of Nichrome wire wrapped around a ceramic core, that glows when an electrical current is passed through it. A Globar, which is a silicon carbide rod across which a voltage is applied,

also can be used as a more intense source. Older spectrometers used sodium chloride prisms to disperse the radiation into monochromatic components, but more modern instruments use a diffraction grating to achieve this effect. Common detectors include the thermocouple detector, whose output voltage varies with temperature changes caused by varying levels of radiation striking the detector. More sensitive detectors include the Golay dectector, in which radiation striking a sealed tube of xenon gas warms the gas and causes pressure changes within the tube, and newer semiconductor detectors whose conductivities vary according to the amount of radiation striking the detector surface.

27.2.1.2 Fourier Transform (FT) Instruments

In Fourier transform (FT) instruments, the radiation is not dispersed, but rather all wavelengths arrive at the detector simultaneously and a mathematical treatment, called a Fourier transform, is used to convert the results into a typical IR spectrum. Instead of a monochromator, the instrument uses an interferometer, which splits an IR beam and then recombines it by reflecting back the split beams with mirrors (Fig. 27-2). As the pathlength of one beam is varied by moving its mirror, the two beams will interfere either constructively or destructively as they are combined, depending on their phase difference. Therefore, the intensity of the radiation reaching the detector varies as a function of the optical path difference, and the pattern of energy intensity obtained as a function of optical path difference is referred to as an interferogram. When a sample is placed in the recombined beam ahead of the detector, the molecules in the sample absorb at their characteristic frequencies, and thus the radiation reaching the detector is modified by the presence of the sample. This interferogram showing intensity versus pathlength is
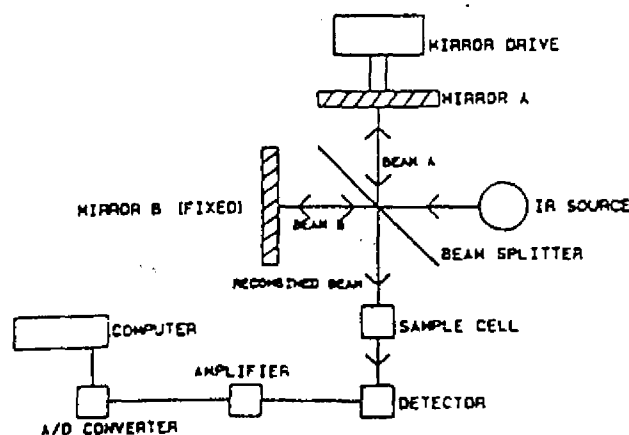
27-2 figure Block diagram of an interferometer and associated electronics typically used in an FTIR instrument.

then converted by Fourier transformation into an IR spectrum giving absorbance versus frequency. A computer allows the mathematical transformation to be completed rapidly. Because all wavelengths are measured at once, FT instruments can acquire spectra more rapidly, with a greatly improved signal-to-noise ratio, as compared to dispersive instruments.

27.3.1.3 Sample Handling Techniques

Liquids are most commonly measured by transmission IR spectroscopy, using cells with a pathlength of 0.01–1.0 mm. Because quartz and glass absorb in the mid-IR region, cell windows of halide or sulfide salts are most commonly used. Since many of these materials are soluble in water, care must be taken when selecting cells for use with aqueous samples. Transmission spectra of solids can be obtained by finely grinding a small amount of the sample with potassium bromide, pressing the mixture into a pellet under high pressure, and inserting the pellet into the IR beam. An alternative technique is to disperse a finely divided solid in Nujol mineral oil to form a mull. Also, attenuated total reflectance (ATR) cells are available for obtaining spectra from solid samples. ATR measures the total amount of energy reflected from the surface of a sample in contact with an IR transmitting crystal. The radiation penetrates a short distance into the sample before it is reflected back in the transmitting medium; therefore, the intensity of the reflected radiation is decreased at wavelengths where the sample absorbs radiation, allowing an absorption spectrum to be obtained. Similarly, internal reflectance cells also are available for use with liquid samples, where the IR radiation penetrates a few micrometers into the liquid before being reflected back into an IR transmitting crystal in contact with the liquid. These types of cells are especially useful for samples such as aqueous liquids that absorb strongly in the mid-IR region. A recent development is the coupling of a microscope to an FTIR spectrometer. The IR beam can be focused through a microscope onto a thin specimen mounted on a microscope slide. The IR spectrum then can be obtained from a very small area of the sample that measures only a few micrometers on each side. By moving the microscope stage, a profile of spectra across the sample can be obtained and used to evaluate the homogeneity of the sample.

Transmission spectra can be obtained from gas samples using a sealed 2–10 cm glass cell with IR transparent windows. For trace analysis, multiple-pass cells are available that reflect the IR beam back and forth through the cell many times to obtain pathlengths as long as several meters. FTIR instruments also can be interfaced to a gas chromatograph, to obtain spectra of compounds eluting from the chromatography column.

27.3.2 Applications of Mid-IR Spectroscopy

27.3.2.1 Absorption Bands of Organic Functional Groups

The wavelength bands where a number of important organic functional groups absorb radiation in the mid-IR region are shown in Table 27-1.

27.3.2.2 Presentation of Mid-IR Spectra

Spectra are normally presented with either wavenumbers or wavelengths plotted on the x-axis and either percent transmittance or absorbance plotted on the y-axis. The mid-IR spectrum of polystyrene is shown in Fig. 27-3 and is typical of the common method of presentation of IR spectra.

27.3.2.3 Qualitative Applications

The center frequencies and relative intensities of the absorption bands can be used to identify specific functional groups present in an unknown substance. A substance can also be identified by comparing its mid-IR spectrum to a set of standard spectra and determining the closest match. Spectral libraries are available from several sources, but probably the largest collection of standards is the Sadtler Standard Spectra (Sadtler Division of Bio-Rad, Inc., Philadelphia, PA). Standard spectra are now commonly stored in digital format to allow searching by computer algorithm to determine the best match with an unknown compound. Common food applications include the identification of flavor and aroma compounds, particularly when FTIR measurements are coupled with gas chromatography. IR spectra also are useful for obtaining positive identification of packaging films.

27.3.2.4 Quantitative Applications

IR spectroscopic measurements obey Beer's law, although deviations may be greater than in UV-Vis spectroscopy due to the low intensities of IR sources, the low sensitivities of IR detectors, and the relative narrowness of mid-IR absorption bands. However, quantitative measurements can be successfully made. Perhaps the most extensive use of this technique is in the **Infrared Milk Analyzers**, which have the ability to analyze hundreds of samples per hour. The fat, protein, and lactose contents of milk can be determined simultaneously with one of these instruments. The ester carbonyl groups of lipid absorb at 5.73 μm (1742 cm^{-1}), the amide groups of protein at 6.47 μm (1348 cm^{-1}), and the hydroxyl groups of lactose at 9.61 μm (1046 cm^{-1}). These automated instruments homogenize the milk fat globules to minimize light scattering by the sample,

27-1 table — Mid-IR Absorption Frequencies of Various Organic Functional Groups

Group	Absorbing Feature	Frequency (cm^{-1})
Alkanes	—CH stretch and bend	3000–2800
	—CH_2 and —CH_3 bend	1470–1420 and 1380–1340
Alkenes	Olefinic —CH stretch	3100–3000
Alkynes	Acetylenic —CH stretch	3300
Aromatics	Aromatic — CH stretch	3100–3000
	—C═C— stretch	1600
Alcohols	—OH stretch	3600–3200
	—OH bend	1500–1300
	C—O stretch	1220–1000
Ethers	C—O asymmetric stretch	1220–1000
Amines	Primary and secondary — NH stretch	3500–3300
Aldehydes and ketones	—C═O stretch	1735–1700
	—CH (doublet)	2850–2700
Carboxylic acids	—C═O stretch	1740–1720
Amides	—C═O stretch	1670–1640
	—NH stretch	3500–3100
	—NH bend	1640–1550
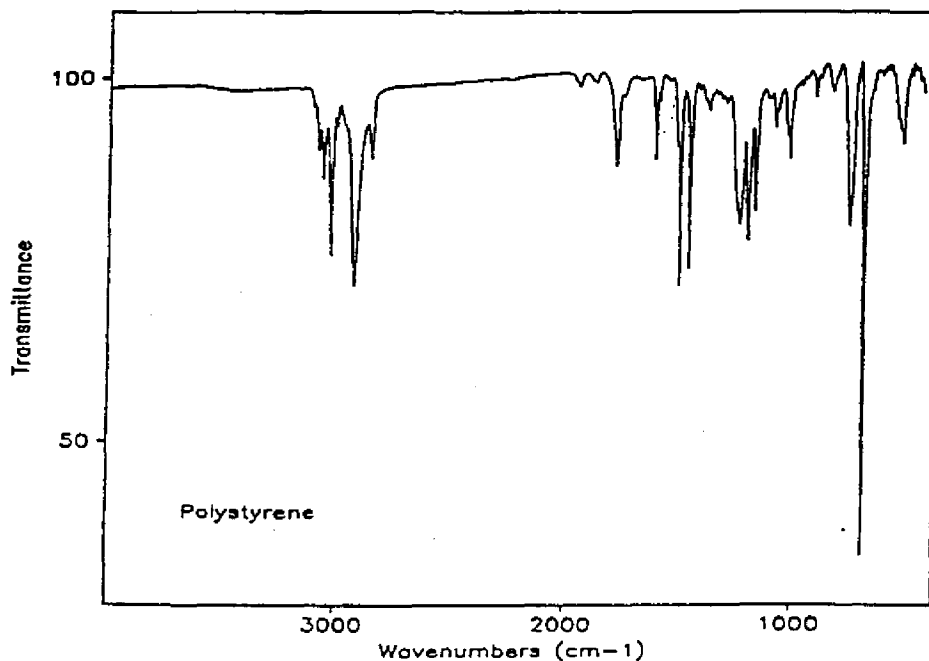
27-3 figure

Mid-IR spectrum of polystyrene, showing percent transmittance versus frequency in wavenumbers. The absorption bands just above 3000 cm^{-1} and at 1600 cm^{-1} indicate the presence of aromatic ring structures in the molecule, while the —CH bands just below 3000 cm^{-1} indicate that saturated hydrocarbon regions also are present.

and then pump the milk into a flow-through cell through which the infrared beam is passed. In some instruments, the monochromator uses simple optical interference filters that pass only a single wavelength of radiation through the sample, and the filter is selected depending on which constituent the operator wishes to measure. The instrument is calibrated using samples of known concentration to establish the slope and intercept of a Beer's law plot. Newer analyzers use an FTIR instrument to measure the absorbance at all

wavelengths simultaneously, and then use a multiple linear regression equation to predict the concentration of each constituent from the absorbance values at selected wavelengths. Multiple linear regression is described in more detail in the section on near-IR spectroscopy. Official methods have been adopted for the IR milk analyzers, and specific procedures for operation of these instruments are given (1, 2).

Commercial instruments also are available for measuring the fat content of emulsified meat samples

by IR spectroscopy. Other quantitative applications include measurement of the degree of unsaturation and cis and trans contents in fats and oils (3).

27.4 NEAR-INFRARED (NIR) SPECTROSCOPY

Measurements in the near-IR (NIR) spectral region (0.7–2.5 μm, equal to 700–2500 nm) are more widely used for quantitative analysis of foods than are mid-IR measurements. Several commercial instruments are available for compositional analysis of foods using NIR spectroscopy. A major advantage of NIR spectroscopy is its ability to measure directly the composition of solid food products by use of diffuse reflectance techniques.

27.4.1 Principles

27.4.1.1 Principles of Diffuse Reflectance Measurements

When radiation strikes a solid or granular material, part of the radiation is reflected from the sample surface. This mirrorlike reflectance is called specular reflectance, and gives little useful information about the sample. Most of the specularly reflected radiation is directed back toward the energy source. Another portion of the radiation will penetrate through the surface of the sample and be reflected off several sample particles before it exits the sample. This is referred to as diffuse reflectance, and this diffusely reflected radiation emerges from the surface at random angles through 180°. Each time the radiation interacts with a sample particle, the chemical constituents in the sample can absorb a portion of the radiation. Therefore, the diffusely reflected radiation contains information about the chemical composition of the sample, as indicated by the amount of energy absorbed at specific wavelengths.

The amount of radiation penetrating and leaving the sample surface is affected by the size and shape of the sample particles. Therefore, if solid or granular materials are to be ground prior to analysis by NIR reflectance spectroscopy, it is desirable to use a sample preparation mill that yields a fine particle size with a uniform size distribution. Mills such as the Udy Cyclotec (Boulder, CO), equipped with a 1 mm screen, are useful for preparing granular materials, such as cereal grains, for analysis by NIR reflectance techniques.

27.4.1.2 Absorption Bands in the NIR Region

The absorption bands observed in the NIR region are primarily overtones. Therefore, the absorptions tend to

be weak in intensity. However, this is actually an advantage, since absorption bands that have sufficient intensity to be observed in the NIR region arise primarily from functional groups that have a hydrogen atom attached to a carbon, nitrogen, or oxygen, which are common groups in the major constituents of food such as water, proteins, lipids, and carbohydrates. Table 27-2 lists the absorption bands associated with a number of important food constituents.

The absorption bands in the NIR region tend to be broad and frequently overlap, yielding spectra that are quite complex. However, these broad bands are especially useful for quantitative analysis. Typical NIR spectra of wheat, dried egg white, and cheese are shown in Fig. 27-4. Note that strong absorption bands associated with the —OH groups of water are centered at ca. 1450 and 1940 nm. These bands are the dominant features in the spectrum of cheese, which contains 30–40% moisture, and they are still prominent even in the lower moisture wheat and egg white samples. Bands arising from the —NH groups in protein can be observed at 2060 nm and 2180 nm in the egg white spectrum but are partially obscured by a starch absorption band, centered at 2100 nm, in the wheat sample. Relatively sharp absorption bands arising from —CH groups in lipid can be observed at 2310 and 2350 nm, and another band from these groups is seen around 1730 nm. The 1730 nm band overlaps a weak protein absorption. The lipid bands are distinctly observable in the cheese spectrum.

27.4.2 Instrumentation

A commercial NIR spectrometer is shown in Fig. 27-5. The radiation source in most NIR instruments is a tungsten-halogen lamp with a quartz envelope, similar to a projector lamp. These lamps emit significant amounts of radiation in both the visible and NIR spectral regions. Semiconductor detectors are most commonly used in NIR instruments, with silicon detectors used in the 700–1100 nm range, and lead sulfide used in the 1100–2500 nm region. Most commercial NIR instruments use monochromators, rather than interferometers, although some recently manufactured commercial instruments are now using Fourier transform technology. Some monochromator-based instruments use diffraction gratings that allow measurements to be taken at each wavelength over the entire NIR spectral region. Other instruments are dedicated to specific applications and use optical interference filters to select 6–20 discrete wavelengths that can be impinged on the sample. The filters are selected to obtain wavelengths that are known to be absorbed by the sample constituents. The instrument inserts filters one at a time into the light beam to direct individual wavelengths of radiation onto the sample.

27-2
table   Near-IR Absorption Bands of Various Food Constituents

Constituent	Absorber	Wavelength (nm)
Water	—OH stretch/deformation combination	1920–1950
	—OH stretch	1400–1450
Protein—peptides	—NH deformation	2080–2220 and 1560–1670
Lipid	Methylene —CH stretch	2300–2350
	—CH$_2$ and —CH$_3$ stretch	1680–1760
Carbohydrate	C—O, O—H stretching combination	2060–2150
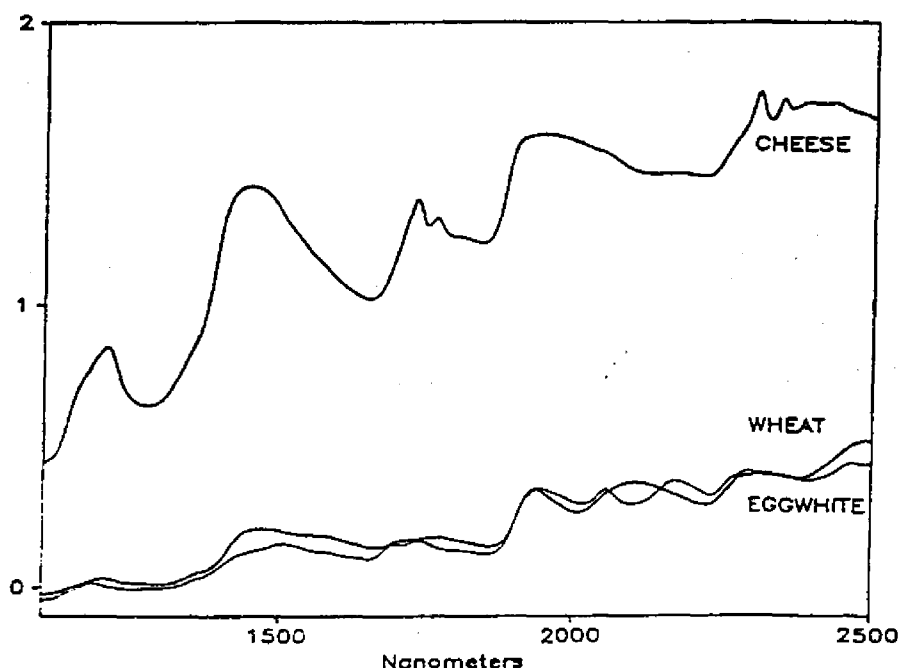
27-4
figure   NIR spectra of cheese, wheat, and dried egg white plotted as log(1/R) versus wavelength in nm.

Either reflectance or transmittance measurements may be made in NIR spectroscopy, depending on the type of sample. In the reflectance mode, used primarily for solid or granular samples, it is desirable to measure only the diffuse reflectance that contains information about the sample. In some instruments, this is accomplished by positioning the detectors at a 45° angle with respect to the incoming infrared beam, so that the specularly reflected radiation is not measured (Fig. 27-6a). Other instruments use an integrating sphere, which is a gold-coated metallic sphere with the detectors mounted inside (Fig. 27-6b). The sphere collects the diffusely reflected radiation coming at various angles from the sample and focuses it onto the detectors. The specular component escapes from the sphere through the same port by which the incident beam enters and strikes the sample.

Most samples are prepared by packing the food tightly into a cell against a quartz window, thereby providing a smooth, uniform surface from which reflection can occur. Quartz does not absorb in the NIR region. At each wavelength, the intensity of light reflecting from the sample is compared to the intensity reflected from a nonabsorbing reference, such as a ceramic or fluorocarbon material or the interior of the integrating sphere. Reflectance (R) is calculated by the following formula:

$$R = I/I_0 \qquad [4]$$

where:

I = the intensity of radiation reflected from the sample at a given wavelength

I_0 = the intensity of radiation reflected from the reference at the same wavelength
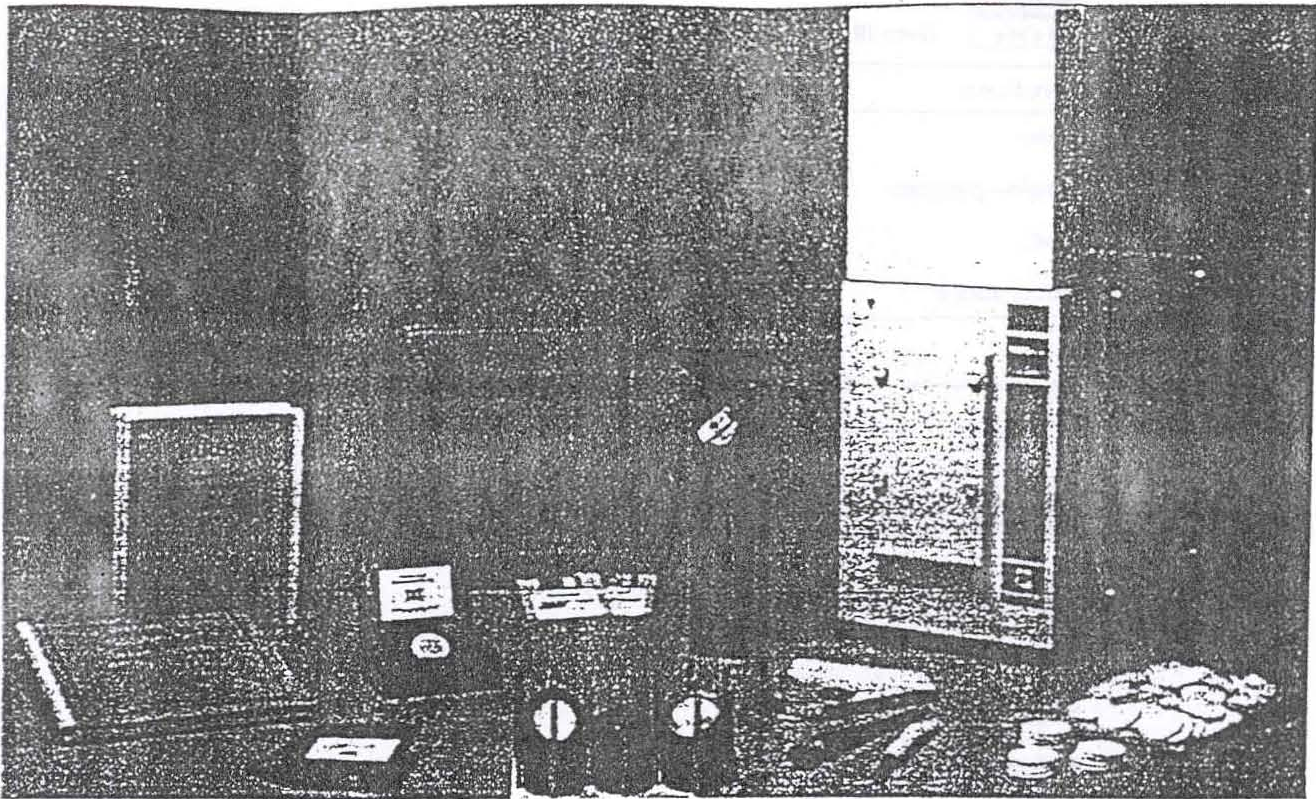
27-5 figure A modern commercial NIR spectrometer. (Courtesy of Foss/NIRSystems Inc., Silver Springs, MD.)

(a)　　　　　　　　　(b)
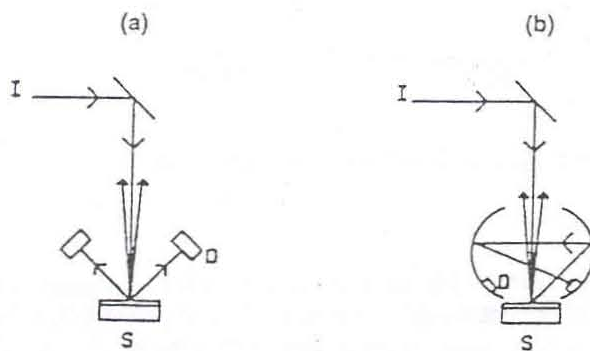
27-6 figure Typical instrument geometries for measuring diffuse reflectance from solid food samples. Radiation from the monochromator (I) is directed by a mirror onto the sample (S). Diffusely reflected radiation is measured directly by detectors (D) placed at a 45° angle to the incident beam (a) or is collected by an integrating sphere and focused onto the detectors (b). In both cases, the specularly reflected radiation is not measured.

Reflectance data are expressed most commonly as log $(1/R)$, an expression analogous to absorbance in transmission spectroscopy. Reflectance measurements also are expressed sometimes as differences, or derivatives, of the reflectance values obtained from adjacent wavelengths:

$$(\log R_2 - \log R_1) \qquad [5]$$

or

$$(2 \log R_2 - \log R_1 - \log R_3) \qquad [6]$$

These derivative values are measures of the changes in slope of the spectrum.

Transmission measurements also can be made in the NIR region, and this is usually the method of choice for liquid samples. A liquid is placed in a quartz cuvette and the absorbance measured at the wavelengths of interest. Transmission measurements also can be taken from solid samples, but generally only in the 700–1100 nm range. In this wavelength region, the absorption bands are higher overtones that are very weak, allowing the radiation to penetrate through several millimeters of a solid sample. The use of transmission measurements can minimize the degree of sample preparation needed. Since the IR beam passes through the entire sample, the need for a smooth, homogeneous sample surface is reduced.

27.4.3 Quantitative Methods Using NIR Spectroscopy

NIR instruments can be calibrated to measure various constituents in food and agricultural commodities.

Because of the overlapping nature of the NIR absorption bands, it is usually necessary to take measurements at two or more wavelengths to quantitate a food component reliably. The instrument uses an equation of the following form to predict the amount of a constituent present in the food from the spectral measurements:

$$
\begin{aligned}
\% \text{ constituent} = z &+ a \log(1/R_1) \\
&+ b \log(1/R_2) \\
&+ c \log(1/R_3) + \ldots \ldots \quad [7]
\end{aligned}
$$

where each term represents the spectral measurement at a different wavelength multiplied by a corresponding coefficient. Each coefficient and the intercept (z) are determined by multivariate regression analysis. Absorbance or derivatized reflectance data also can be used in lieu of the $\log(1/R)$ format. Use of derivatized reflectance data has been found to provide improved results in some instances, particularly with samples that may not have uniform particle sizes.

27.4.3.1 Calibration Methods Using Multiple Linear Regression

The first step in calibrating an NIR instrument is to select a set of calibration, or training, samples. The samples should be representative of the products that will be analyzed, contain the constituent of interest at levels covering the range that is expected to be encountered, and have a relatively uniform distribution of concentrations across that range. The calibration samples are analyzed by the classical analytical method normally used for that constituent, and spectral data also are obtained on each sample with the NIR instrument at all available wavelengths. All data are stored into computer memory. Multiple linear regression is then most commonly used to select the optimum wavelengths for measurement and the associated coefficients for each wavelength. Wavelengths are selected based on statistical significance by using a step forward or reverse stepwise regression procedure or by using a computer algorithm that tests regressions using all possible combinations of two, three, or four wavelengths to determine the combination that provides the best results. Most calibrations will use between two and six wavelengths, and one should always check to make certain that the wavelengths chosen on the basis of statistical significance also make sense from a spectroscopic standpoint. Calibration results are evaluated by comparing the multiple correlation coefficients, Fs of regression, and standard errors for the various equations developed. It is desirable to maximize the correlation coefficient (generally R should be >0.9) and minimize the standard error. A calibration always should be tested by using the instru-

ment to predict the composition of a set of test samples that are completely independent of the calibration set and comparing the results obtained to the classical method.

27.4.3.2 Calibration Development Using Full Spectrum Methods

Recently, calibration techniques such as partial least squares (PLS) regression and principal components regression (PCR) have been developed that use information from all wavelengths in the entire NIR spectrum, rather than a few selected wavelengths, to predict sample composition. PLS and PCR use data reduction techniques to extract from a large number of variables (i.e., reflectance or absorbance measurements at many wavelengths) a much smaller number of new variables that account for most of the variability in the samples. These new variables then can be used to develop a regression equation to predict the amount of a constituent in samples of a food. In PLS and PCR methods, no spectral information is eliminated, as it is when measurements at only a limited number of wavelengths are used. PLS and PCR methods are reported to yield improved results for some samples (4).

27.4.4 Qualitative Analysis By NIR Spectroscopy

NIR spectroscopy also can be used to classify a sample into one of two or more groups, rather than to provide quantitative measurements. Discriminant analysis techniques can be used to compare the NIR spectrum of an unknown sample to the spectra of samples from different groups. The unknown sample then is classified into the group to which its spectrum is most similar. While this technique has been more widely used in the chemical and pharmaceutical industries for raw material identification, it is beginning to be used for food applications, including the classification of skim milk powders based on level of heat treatment (5), the classification of wheat as hard red spring or hard red winter (6), and the identification of orange juice samples from different sources (7).

27.4.5 Applications of NIR Spectroscopy to Food Analysis

Theory and applications of NIR spectroscopy to food analysis have been discussed in several publications (8–10). The technique has found its widest use in the grain, cereal products, and oilseed processing industries. NIR techniques using reflectance measurements from ground or powdered samples have been adopted as approved methods of analysis by the American Association of Cereal Chemists (11) for measuring pro-

tein in barely, oats, rye, triticale, and wheat (Method 39-10), protein in wheat flour (Method 39-11), and protein and oil in soybeans (Method 39-20). Techniques using measurements from whole kernel grains have also been approved for protein, oil, and moisture in soybeans (Method 39-21), and protein in wheat (Method 39-25). NIR reflectance measurements also have been approved for estimating wheat hardness (Method 39-70A). These approved methods describe the instruments available for use in making these measurements, and the proper techniques for preparing samples and calibrating instruments. NIR instruments now are used by the official grain inspection agencies in both the United States and Canada for measuring protein, moisture, and oil in cereals and oilseeds.

NIR spectroscopy also can be used for numerous other commodities and food products. The technique has been used successfully to measure moisture, protein, and fat in red meats and processed meat products (12–14), poultry, and fish (15). NIR spectroscopy is useful also for analyzing a number of dairy products, including measuring moisture and fat in butter; moisture, fat, and protein in cheese (16, 17); and lactose, protein, and moisture in milk and whey powders (18). In addition, moisture, fat, and protein have been determined in dehydrated eggs using NIR reflectance measurements (19). NIR techniques also have shown promise for measuring total sugars and soluble solids in fruits and vegetables (20, 21), and are being used commercially for monitoring the sugar content in corn sweeteners (22).

NIR spectroscopy also is showing potential for measuring specific chemical constituents in a food that affect its end-use quality, for directly predicting processing characteristics of a commodity that are related to its chemical composition, and for monitoring changes that occur during processing. Examples include determining the amylose content in rice starch, an important determinant of rice quality, by both reflectance and transmission measurements (23, 24); predicting corn processing quality (25, 26); monitoring the degree of cook obtained during extrusion processing of wheat products (27); and monitoring the coagulation of milk during cheese making (28).

These are some examples of current applications, but if a substance absorbs in the NIR region, and is present at a level of a few tenths of a percent or greater, it has potential for being measured by this technique. The primary advantage of NIR spectroscopy is that once the instrument has been calibrated, several constituents in a sample can be measured rapidly (from 30 sec to 2 min) and simultaneously. To measure multiple constituents, a calibration equation for each constituent is stored into the memory of the instrument. Measurements are taken at all wavelengths needed by the calibrations, and each equation then is solved to predict the constituents of interest. No sample weigh-

ing is required, and the NIR technique can be used by employees without extensive training. It also is applicable for on-line measurement systems. Disadvantages include the high initial cost of the instrumentation, which may require a large sample load to justify the expenditure, and the fact that specific calibrations must be developed for each product to be measured. Also, the results produced by the instrument can be no better than the data used to calibrate it, which makes careful analysis of the calibration samples of highest importance.

27.5 SUMMARY

IR spectroscopy measures the absorption of radiation in the near- ($\lambda = 0.8$–2.5 μm) or mid- ($\lambda = 2.5$–15 μm) IR regions by molecules in food or other substances. IR radiation is absorbed as molecules change their vibrational energy levels. Mid-IR spectroscopy is especially useful for qualitative analysis, such as identifying specific functional groups present in a substance. Different functional groups absorb different frequencies of radiation, allowing the groups to be identified from the spectrum of a sample. Quantitative analysis also can be achieved by mid-IR spectroscopy, with milk analysis being a major application. NIR spectroscopy is used most extensively for quantitative applications, using either transmission or diffuse reflectance measurements, that can be taken directly from solid foods. By using multivariate statistical techniques, NIR instruments can be calibrated to measure the amounts of various constituents in a food sample based on the amount of IR radiation absorbed at specific wavelengths. NIR spectroscopy requires much less time to perform quantitative analysis than do many conventional wet chemical or chromatographic techniques.

27.6 STUDY QUESTIONS

1. Describe the factors that affect the frequency of vibration of a molecular functional group and thus the frequencies of radiation that it absorbs. Also, explain how the fundamental absorption and overtone absorptions of a molecule are related.

2. Describe the essential components of a Fourier transform (FT) mid-IR spectrometer and their function, and compare the operation of the FT instrument to a dispersive instrument. What advantages do Fourier transform instruments have over dispersive IR spectrophotometers?

3. Of the three antioxidants BHT (butylated hydroxytoluene), BHA (butylated hydroxyanisole), and propyl gallate, which would you expect to have a strong IR absorption band in the 1700–1750 cm^{-1} spectral region? Look up these compounds in a reference book if you are uncertain of their structure.

4. Describe the two ways in which radiation is reflected from a solid or granular material. Which type of reflected radiation is useful for making quantitative measurements on

solid samples by near-infrared (NIR) spectroscopy? How are NIR reflectance instruments designed to select for the desired component of reflected radiation?

5. Describe the steps involved in calibrating a NIR reflectance instrument to measure the protein content of wheat flour. Why is it usually necessary to make measurements at more than one wavelength?

27.7 REFERENCES

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28

chapter

Atomic Absorption and Emission Spectroscopy

Dennis D. Miller

28.1 INTRODUCTION

Atomic spectroscopy has played a major role in the development of our current database for mineral nutrients and toxicants in foods. When atomic absorption spectrometers became widely available in the sixties and seventies, the development of atomic absorption methods for accurately measuring trace amounts of mineral elements in biological samples paved the way for unprecedented advances in fields as diverse as food analysis, nutrition, biochemistry, and toxicology. The application of plasmas as excitation sources for atomic emission spectroscopy led to the commercial availability of the inductively coupled plasma emission spectrometer beginning in the late seventies. This instrument has further enhanced our ability to measure the mineral composition of foods and other materials rapidly, accurately, and precisely. These two instrumental methods have largely replaced traditional wet chemistry methods for mineral analysis of foods, although traditional methods for iron and phosphorus remain in wide use today (see Chapter 10).

In theory, virtually all of the elements in the periodic chart may be determined by atomic absorption or atomic emission spectroscopy. In practice, atomic spectroscopy is used primarily for the determination of mineral elements. Table 28-1 lists mineral elements of concern in foods. The database for Ca, Fe, Na, and K in foods is reasonably good. The database for the trace

elements and toxic heavy metals is incomplete and should be expanded.

This chapter deals with the basic principles that underlie analytical atomic spectroscopy and provides an overview of the instrumentation available for measuring atomic absorption and emission. In addition, some practical problems associated with the use of the technology are addressed. Readers interested in a more thorough treatment of the topic are referred to two excellent monographs available from the Perkin-Elmer Corporation. One is by Beaty and Kerber (2); the other is by Boss and Fredeen (3).

The following abbreviations will be used throughout the chapter:

AAS: atomic absorption spectroscopy
AES: atomic emission spectroscopy
ICP: inductively coupled plasma

28.2 GENERAL PRINCIPLES

Atomic absorption spectroscopy quantifies the absorption of electromagnetic radiation by well-separated atoms in the gaseous state, while atomic emission spectroscopy measures emission of radiation from atoms excited by heat or other means. Atomic spectroscopy is particularly well suited for analytical measurements because atomic spectra consist of discrete lines, and every element has a unique spectrum. Therefore, individual elements can be identified and quantified with accuracy and precision even in the presence of atoms of other elements.

28.2.1 Energy Transitions in Atoms

Atomic absorption spectra are produced when ground state atoms (or ions) absorb energy from a radiation source. Atomic emission spectra are produced when excited atoms emit energy on returning to the ground state. Absorption of a photon of radiation causes an outer shell electron to jump to a higher energy level, moving the atom into an excited state. The excited atom may fall back to a lower energy state, releasing a photon in the process. Atoms absorb or emit radiation of discrete wavelengths because the allowed energy levels of electrons in atoms are fixed (not random). The energy change associated with a transition between two energy levels is directly related to the frequency of the absorbed radiation:

$$E_e - E_s = h\nu \qquad [1]$$

where:

E_e = energy in excited state

28-1
table

Mineral Elements in Foods Classified According to Nutritional Essentiality, Potential Toxic Risk, and Inclusion in USDA Nutrient Database for Standard Reference

Essential Nutrient	Toxicity Concern	USDA Nutrient Database
Calcium	Lead	Calcium
Phosphorus	Mercury	Iron
Sodium	Cadmium	Magnesium
Potassium	Nickel	Phosphorus
Chlorine	Arsenic	Potassium
Magnesium		Sodium
Iron		Zinc
Iodine		Copper
Zinc		Manganese
Copper		
Selenium		
Chromium		
Manganese		
Arsenic		
Boron		
Molybdenum		
Nickel		
Silicon		

From (1).

E_g = energy in ground state
h = Planck's constant
v = frequency of the radiation

Rearranging, we have:

$$v = (E_e - E_g)/h \qquad [2]$$

or, since $v = c/\lambda$

$$\lambda = hc/(E_e - E_g) \qquad [3]$$

where:

c = speed of light
λ = wavelength of the absorbed or emitted light

The above relationships clearly show that for a given electronic transition, radiation of a discrete wavelength is either absorbed or emitted. Each element has a unique set of allowed transitions and therefore a unique spectrum. The absorption and emission spectra for sodium are shown in Fig. 28-1. For absorption, transitions involve primarily the excitation of electrons in the ground state, so the number of transitions is relatively small. Emission, on the other hand, occurs when electrons in various excited states fall to lower energy levels including, but not limited to, the ground state. Therefore, the emission spectrum has more lines than the absorption spectrum. An energy level diagram for an electron in the 3s orbital of sodium is shown in Fig. 28-2. When a transition is from or to the ground state, it is termed a resonance transition, and the resulting spectral line is called a resonance line. See Chapter 25 for a more detailed discussion of atomic and molecular energy transitions.

28.2.2 Atomization

Atomic spectroscopy requires that atoms of the element of interest be in the atomic state (not combined with other elements in a compound) and that they be well separated in space. In foods, virtually all elements are

present as compounds or complexes and therefore must be atomized before atomic absorption or emission measurements can be made. Atomization involves separating particles into individual molecules (vaporization) and breaking molecules into atoms. It is usually accomplished by exposing the analyte to high temperatures in a flame or plasma although other methods may be used. A solution containing the analyte is introduced into the flame or plasma as a fine mist. The solvent quickly evaporates, leaving solid particles of the analyte that vaporize and decompose to atoms that may absorb radiation (atomic absorption) or become excited and subsequently emit radiation (atomic emission). This process is shown schematically in Fig. 28-3.

Three methods for atomizing samples are summarized in Table 28-2.

28.3 ATOMIC ABSORPTION SPECTROSCOPY (AAS)

Atomic absorption spectroscopy is an analytical method based on the absorption of ultraviolet or visible radiation by free atoms in the gaseous state. It is a relatively simple method and may be accomplished with instruments ranging in price from \$15,000 to \$50,000. It is the most widely used form of atomic spectroscopy in food analysis. Two types of atomization are commonly used in atomic absorption spectroscopy: flame atomization and electrothermal (graphite furnace) atomization.

28.3.1 Principles of Flame Atomic Absorption Spectroscopy

Figure 28-4 shows a simplified diagram of a flame atomic absorption spectrometer.

In flame atomic absorption spectroscopy, a nebulizer-burner system is used to convert a solution of the sample into an atomic vapor. It is important to note that the sample must be in solution (usually an aqueous solution) before it can be analyzed by flame atomic
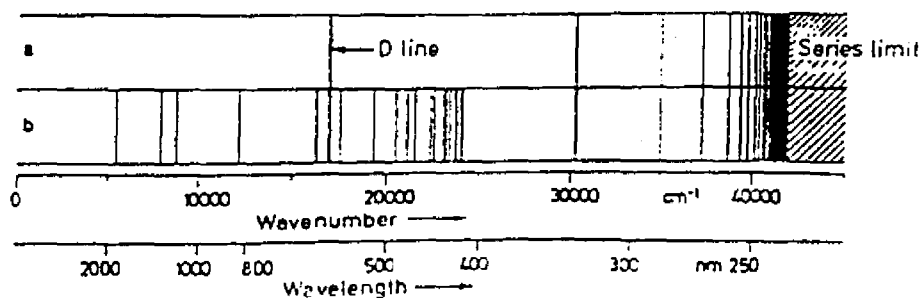
28-1 Spectra for sodium. The upper spectrum (a) is the absorption spectrum and the lower (b) is the emission spectrum. [From (4). Reprinted with permission by VCH Publishers (1985).]
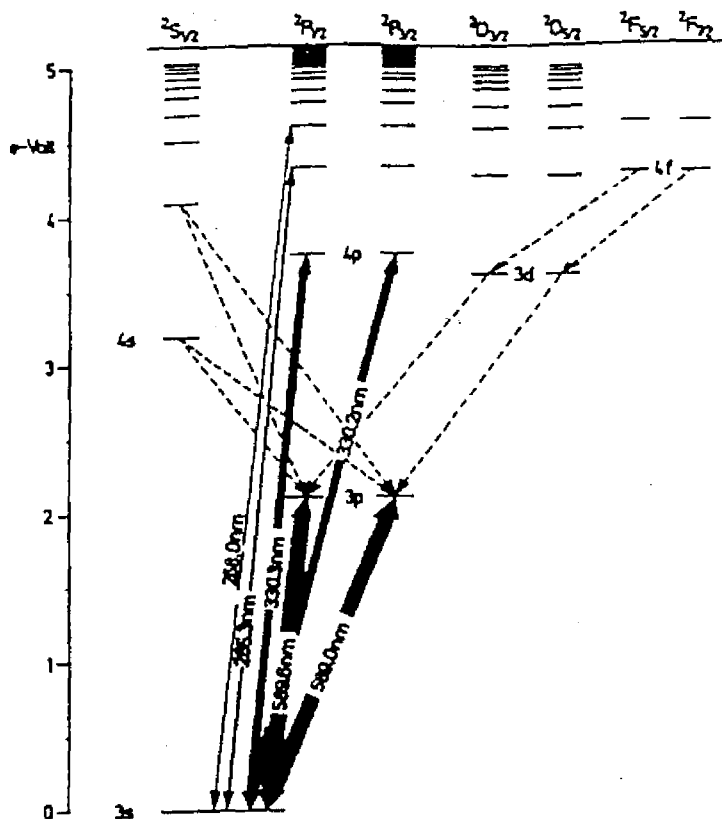
28-2 figure An energy level diagram for sodium, showing transitions between allowed energy levels. The width of the lines is proportional to the intensity of the absorbed or emitted radiation. Solid lines represent transitions allowed in either absorption or emission. Broken lines represent transitions that occur only during emission. [From (4). Reprinted with permission of VCH Publishers (1985).]
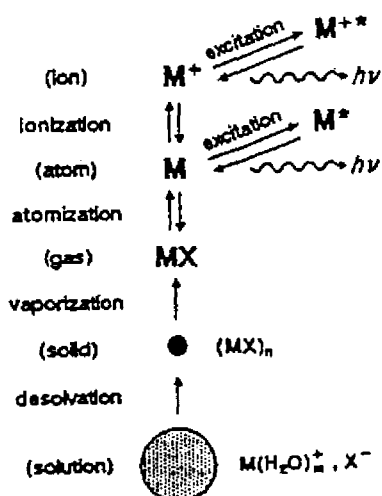
28-3 figure A schematic representation of the atomization of an element in a flame or plasma. The large circle at the bottom represents a tiny droplet of a solution containing the element (M) as part of a compound. [From (3). used with permission. Courtesy of the Perkin-Elmer Corporation, Norwalk, CT.]

28-2 table Methods for Atomization of Analytes

Source of Energy for Atomization	Approximate Atomization Temperature, °C	Analytical Method
Flame	1700–3150	AAS, AES
Electrothermal	1200–3000	AAS (graphite furnace)
Inductively coupled argon plasma	6000–8000	ICP-AES

Adapted from (5)

absorption spectroscopy. The sample solution is nebulized (dispersed into tiny droplets), mixed with fuel and an oxidant, and burned in a flame produced by oxidation of the fuel by the oxidant. Atoms and ions are formed within the hottest portion of the flame as analyte compounds are decomposed by the high temperatures. The flame itself serves as the sample compartment. The temperature of the flame is important
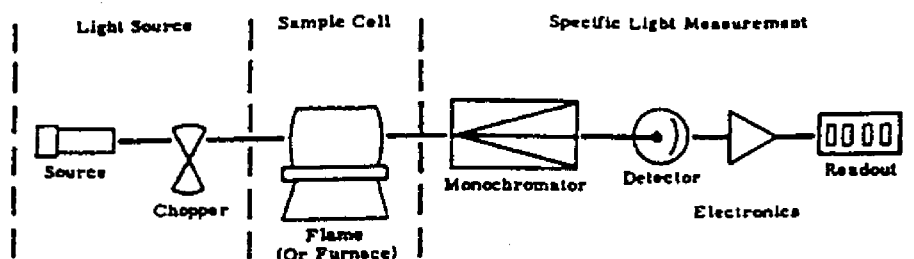
because it will affect the efficiency of converting compounds to atoms and ions and because it influences the distribution between atoms and ions in the flame. Atoms and ions of the same element produce different spectra, and it is desirable to choose a flame temperature that will maximize atomization and minimize ionization. Both atomization efficiency and ionization increase with increasing flame temperature, so choice of the optimal flame is not a simple matter. Flame characteristics may be manipulated by choice of oxidant and fuel and by adjustment of the oxidant/fuel ratio. The most common oxidant–fuel combinations are air–acetylene and nitrous oxide–acetylene. The instrument instruction manual or the literature should be consulted for recommended flame characteristics.

Once the sample is atomized in the flame, its quantity is measured by determining the attenuation of a beam of radiation passing through the flame. For the measurement to be specific for a given element, the radiation source is chosen so that the emitted radiation contains an emission line that corresponds to one of the most intense lines in the atomic spectrum of the element being measured. This is accomplished by fabricating lamps in which the element to be determined serves as the cathode. Thus, the radiation emitted from the lamp is the emission spectrum of the element. The emission line of interest is isolated by passing the beam through a monochromator so that only radiation of a very narrow band width reaches the detector. Usually, one of the strongest spectral lines is chosen; for example, for sodium the monochromator is set to pass radiation with a wavelength of 589.0 nm (see Fig. 28-2). The principle of this process is illustrated in Fig. 28-5. Note that the intensity of the radiation leaving the flame is less than the intensity of radiation coming from the source. This is because sample atoms in the flame absorb some of the radiation. Notice also that the line width of the radiation from the source is narrower than the corresponding line width in the absorption spectrum. This is because the higher temperature of the flame causes a broadening of the line width.

The amount of radiation absorbed by the sample is given by Beer's law:

$$A = \log(I_0/I) = abc \qquad [4]$$

where:

A = absorbance
I_0 = intensity of radiation incident on the flame
I = intensity of radiation exiting the flame
a = molar absorptivity
b = path length through the flame
c = concentration of atoms in the flame

Clearly, absorbance is directly related to the concentration of atoms in the flame.

25.3.2 Principles of Electrothermal Atomic Absorption Spectroscopy (Graphite Furnace AAS)

Electrothermal atomic absorption spectroscopy is identical to flame atomic absorption spectroscopy except for the atomization process. Electrothermal atomization involves heating the sample to a temperature (2000–3000°C) that produces volatilization and atomization. This is accomplished in a tube or cup positioned in the light path of the instrument so that absorbance is determined in the space directly above the surface where the sample is heated. The advantages of electrothermal atomization are that it can accommodate smaller samples than are required for flame atomic absorption and that detection limits are lower. Disadvantages are the added expense of the electrothermal furnace, lower sample throughput, more difficult operation, and lower precision.

25.3.3 Instrumentation for Atomic Absorption Spectroscopy

Atomic absorption spectrometers consist of the following components:

1. Radiation source, usually a hollow cathode lamp

Monochromator
bandwidth

(a)
Emission
spectrum of
source

Radiant power

(b)
Sample
absorption
spectrum

$A = \log \dfrac{P_0}{P}$

Absorbance

(c)
Emission spectrum
after passage
through sample
and monochromator

Radiant power

λ_1 λ_2
Wavelength
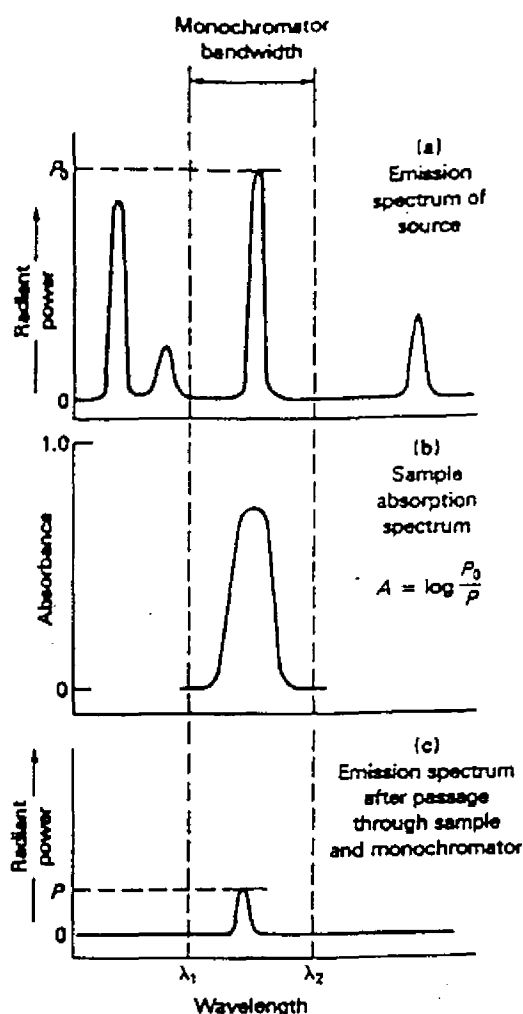
28-5 figure

Schematic representation of the absorption of radiation by a sample during an atomic absorption measurement. The spectrum of the radiation source is shown in (a). As the radiation passes through the sample (b), it is partially absorbed by the element of interest. Absorbance is proportional to the concentration of the element in the flame. The radiant power of the radiation leaving the sample is reduced because of absorption by the sample (c). [From (5), used with permission. Illustration from *Principles of Instrumental Analysis*, 3rd ed., Solutions Manual by Douglas A. Skoog, copyright © 1985 by Saunders College Publishing, reproduced by permission of the publisher.]

2. Atomizer, usually a nebulizer–burner system or an electrothermal furnace
3. Monochromator, usually an ultraviolet-visible (UV-Vis) grating monochromator
4. Detector, usually a photomultiplier tube
5. Readout device, analog or digital

The configuration of a double-beam atomic absorption spectrometer is illustrated in Fig. 28-6. (See

Fig. 28-4 for a diagram of a single-beam instrument. In double-beam instruments, the beam from the light source (hollow cathode lamp) is split by a rotating mirrored chopper into a reference beam and a sample beam. The reference beam is diverted around the sample compartment (flame or furnace) and recombined before passing into the monochromator. The electronics are designed to produce a ratio of the reference and sample beams. This way, fluctuations in the radiation source and the detector are canceled out, yielding a more stable signal.

28.3.3.1 Radiation Source

The radiation source in atomic absorption spectrometers is called a hollow cathode lamp. Hollow cathode lamps consist of a hollow tube filled with argon or neon, an anode made of tungsten, and a cathode made of the metallic form of the element being measured (Fig. 28-7). When voltage is applied across the electrodes, the lamp emits radiation characteristic of the metal in the cathode; if the cathode is made of iron, an iron spectrum is emitted. When this radiation passes through a flame containing the sample, iron atoms in the flame will absorb some of it because it contains radiation of exactly the right energy for exciting iron atoms. This makes sense when we remember that for a given electronic transition, either up or down in energy, the energy of an emitted photon is exactly the same as the energy of an absorbed photon. Of course, this means that it is necessary to use a different lamp for each element analyzed (there are a limited number of multielement lamps available that contain cathodes made of more than one element). Hollow cathode lamps for about 40 metallic elements may be purchased from commercial sources, which means atomic absorption may be used for the analysis of up to 40 elements.

Radiation reaching the monochromator comes from two sources, the attenuated beam from the hollow cathode lamp and excited atoms in the flame. Instruments are designed to discriminate between these two sources either by modulating the lamp so that the output fluctuates at a constant frequency or by positioning a chopper perpendicular to the light path between the source and the flame (Fig. 28-6). A chopper is a disk with segments removed. The disk is rotated at a constant speed so that the light beam reaching the flame is either on or off at regular intervals. The radiation from the flame is continuous. Therefore, the radiation reaching the detector consists of the sum of an alternating and a direct signal. Instrument electronics subtract the direct signal and send only the alternating signal to the readout. This effectively eliminates the contribution of emissions from elements in the flame to the final signal.
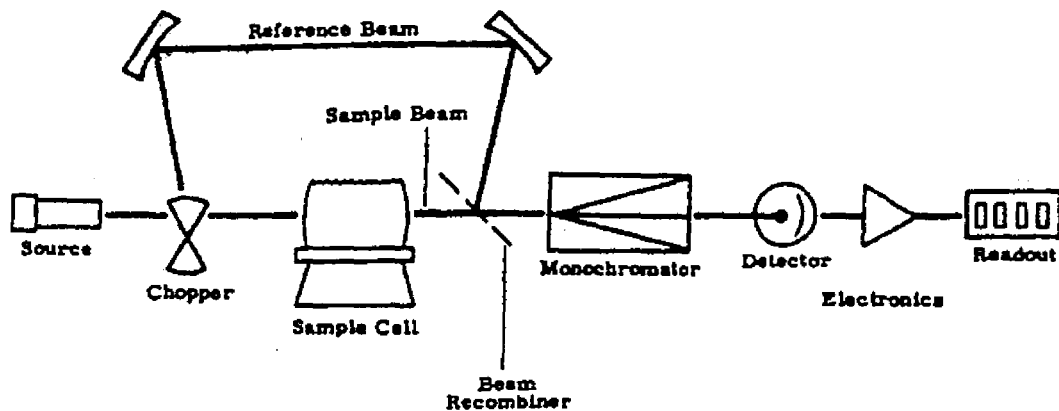
28-6 figure Schematic representation of a double-beam atomic absorption spectrophotometer. [From (2), used with permission. Courtesy of the Perkin-Elmer Corporation, Norwalk, CT.]
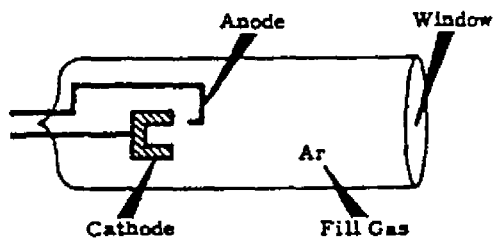
28-7 figure Schematic representation of a hollow cathode lamp. [From (2), used with permission. Courtesy of the Perkin-Elmer Corporation, Norwalk, CT.]

28-8 figure Schematic representation of a nebulizer-burner assembly for an atomic absorption spectrophotometer. [From (2), used with permission. Courtesy of the Perkin-Elmer Corporation, Norwalk, CT.]

28.3.3.2 Atomizers

Several types of atomizers are used in atomic absorption spectroscopy. These include flame, electrothermal, cold vapor technique for mercury, and hydride generation.

The flame atomizer consists of a nebulizer and a burner (Fig. 28-8). The nebulizer is designed to convert the sample solution into a fine mist or aerosol. This is accomplished by aspirating the sample through a capillary into a chamber through which oxidant and fuel are flowing. The chamber contains baffles which remove larger droplets, leaving a very fine mist that is carried into the flame by the oxidant-fuel mixture. The larger droplets fall to the bottom of the mixing chamber and are collected as waste. The burner head contains a long, narrow slot that produces a flame that may be 5–10 cm in length. This gives a long pathlength that increases the sensitivity of the measurement.

Flame characteristics may be manipulated by adjusting oxidant/fuel ratios and by choice of oxidant and fuel. Air-acetylene and nitrous oxide-acetylene are the most commonly used oxidant-fuel mixtures although other oxidants and fuels may be used for some

elements. There are three types of flames: (1) Stoichiometric. This flame is produced from stoichiometric amounts of oxidant and fuel so the fuel is completely burned and the oxidant is completely consumed. It is characterized by yellow fringes. (2) Oxidizing. This flame is produced from a fuel-lean mixture. It is the hottest flame and has a clear blue appearance. (3) Reducing. This flame is produced from a fuel-rich mixture. It is a relatively cool flame and has a yellow color. Analysis should follow guidelines for the proper type of flame for each element.

Flame atomizers have the advantage of being stable and easy to use. However, sensitivity is relatively low because much of the sample never reaches the flame and the residence time of the sample in the flame is short.

Electrothermal atomizers are typically cylindrical graphite tubes connected to an electrical power supply.

They are commonly referred to as graphite furnaces. The sample is introduced into the tube through a small hole using a microliter syringe (sample volumes normally range from 0.5 to 10 μl). During operation, the system is flushed with an inert gas to prevent the tube from burning and to exclude air from the sample compartment. The tube is heated electrically. Through a stepwise increase in temperature, first the sample solvent is evaporated, then the sample is ashed, and finally the temperature is rapidly increased to 2000-3000°C to rapidly vaporize and atomize the sample.

The cold vapor technique works only for mercury, because mercury is the only element that can exist as free atoms in the gaseous state at room temperature. In this technique, mercury compounds in a sample are reduced to elemental mercury by the action of a strong reducing agent. The elemental mercury is then carried in a stream of air or argon into an absorption cell and atomic absorption is measured the same way as it is in flame ionization and electrothermal instruments. This method has the advantage of very high sensitivity because all of the mercury in the sample can be transferred to the absorption cell and measured. See reference (2) for a more detailed description of this technique.

In the hydride generation technique, volatile hydrides of elements are formed by reacting samples with sodium borohydride. The hydrides then are carried into an absorption cell and heated to decompose them into free atoms. Then atomic absorption measurements are carried out in the same manner as with other atomization techniques. As with the cold mercury vapor technique, sensitivity is high because there is very little sample loss. However, this technique is limited to a relatively few elements that are capable of forming volatile hydrides. These include As, Pb, Sn, Bi, Sb, Te, Ge, and Se. See reference (2) for a more detailed explanation of this techniques.

28.3.3.3 Monochromator

The monochromator is positioned in the optical path between the flame or furnace and the detector (Fig. 28-6). Its purpose is to isolate the resonance line of interest from the rest of the radiation coming from the flame or furnace and the lamp so that only radiation of the desired wavelength reaches the detector. Typically, monochromators of the grating type are used. (See Chapter 25.)

28.3.3.4 Detector/Readout

The detector is a photomultiplier tube (PMT) that converts the radiant energy reaching it into an electrical signal. This signal is processed to produce either an analog or a digital readout. Modern instruments may

be interfaced with computers for data collection, manipulation, and storage. (See Chapter 25.)

28.4 ATOMIC EMISSION SPECTROSCOPY (AES)

In contrast to atomic absorption spectroscopy, the source of radiation in atomic emission spectrometers is the excited atoms or ions in the sample rather than an external source. Figure 28-9 shows a simplified diagram of an atomic emission spectrometer. As with atomic absorption spectroscopy, the sample must be atomized to produce usable spectra for quantitative analysis. The difference is that in emission spectroscopy, sufficient heat is applied to the sample to excite atoms to higher energy levels. Aside from the external radiation source required for atomic absorption spectroscopy, instrumentation for atomic emission spectroscopy is similar. In fact, many instruments may be operated in either the absorption or emission mode.

Emissions are produced when electrons in excited atoms fall back to lower energy states. Emissions have wavelengths characteristic of individual elements because, as discussed previously, the allowed energy levels for electrons are unique for each element. Energy for excitation may be produced by several methods, including heat (usually from a flame), light (from a laser), electricity (arcs or sparks), or radio waves (inductively coupled plasma) (6). Emissions are passed through monochromators or filters prior to detection by photomultiplier tubes or charge injection devices.

The two most common forms of atomic emission spectroscopy used in food analysis are flame emission spectroscopy and inductively coupled plasma (ICP) atomic emission spectroscopy.

28.4.1 Principles of Flame Emission Spectroscopy

Flame emission spectroscopy employs a nebulizer-burner system to atomize and excite the sample. The instrument may be either a spectrophotometer (which uses a monochromator to isolate desired emission line) or a photometer (which uses a filter to isolate emission

Flame or
Plasma   Monochromator   Detector

28-9
figure

A simplified diagram of an atomic emission spectrometer. [From (3), used with permission. Courtesy of the Perkin-Elmer Corporation, Norwalk, CT.]

lines). Flame emission is most useful for elements with relatively low excitation energies. These include sodium, potassium, and calcium.

28.4.2 Principles of Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES)

Inductively coupled plasma emission spectroscopy has become widely available only in the last decade or so. In ICP-AES, a plasma is used as the atomization–excitation source. A plasma is defined as gaseous mixture containing significant concentrations of cations and electrons. Temperatures in plasmas are very high (in the neighborhood of 8000–10,000 K) resulting in very effective atomization. Even so, excessive ionization of sample atoms is not a problem, probably because of the high concentration of electrons contributed by the ionization of the argon.

28.4.3 Instrumentation for Flame Emission Spectroscopy

Flame emission spectrometers consist of the following components:

1. Atomization–excitation source, usually a nebulizer–laminar flow burner
2. Monochromator or filter. Instruments with monochromators are more versatile because any wavelength in the UV-Vis spectrum can be selected. Instruments designed for routine analysis of alkali and alkaline earth metals may employ interference filters to isolate the desired emission line.
3. Detector
4. Readout device

A comparison of the components of atomic absorption and flame emission spectrometers quickly reveals their similarities. In emission spectrometers, the flame is the radiation source, so the hollow cathode lamp and the chopper are not required. Many modern atomic absorption instruments also can be operated as flame emission spectrometers. Specialized instruments specifically designed for the analysis of sodium, potassium, lithium, and calcium in biological samples are made by some manufacturers. These instruments are called flame photometers. They employ interference filters to isolate the spectral region of interest. Low flame temperatures are used so that only easily excited elements such as the alkali and alkaline earth metals produce emissions. This results in a simpler spectrum

and reduces interference from other elements that may be present.

28.4.4 Instrumentation for ICP-AES

There are three basic types of ICP-AES instruments available today: the simultaneous PMT spectrometer, the sequential PMT spectrometer, and the charge injection device (CID) spectrometer. All three instruments are capable of determining multiple elements in the same sample. The PMT spectrometers use photomultiplier tubes as detectors. The CID spectrometer uses a charge injection device as the detector. The simultaneous PMT spectrometer determines a limited number of elements simultaneously, while the sequential PMT spectrometer determines multiple elements sequentially in rapid succession. The CID spectrometer also measures multiple elements simultaneously but it can detect a much larger number of elements than instruments with a PMT detector.

Inductively coupled plasma atomic emission spectrometers consist of the following components (see Fig. 28-10):

1. Argon plasma torch
2. Monochromator or polychromator
3. Detector(s): one photomultiplier tube for sequential spectrometers, multiple photomultiplier tubes for simultaneous PMT spectrometers, a CID detector for CID spectrometers
4. Computer for data collection and treatment

28.4.4.1 Argon Plasma Torch

The development of the inductively coupled argon plasma torch was a major advance in the field of quantitative atomic emission spectroscopy. The ICP torch is shown schematically in Fig. 28-11. It consists of three concentric quartz tubes. The open (top) end of the outermost tube is encased in the induction coil, which is connected to a radiofrequency generator. Argon gas flows through the three tubes during operation. The torch is started by ionizing the argon gas with a spark from a Tesla discharge. The oscillating magnetic field generated by the radiofrequency induction coil couples with the argon ions and electrons inside the torch, causing them to accelerate in an annular path. The ions and electrons collide with argon atoms, causing heat generation and further ionization of the argon. The resulting plasma reaches temperatures of 6000–10,000 K. To prevent the quartz tube from melting, a stream of argon (or nitrogen) is directed tangentially upward along the inside of the outer quartz tube. This cools the tube and isolates the plasma in the center of the tube.
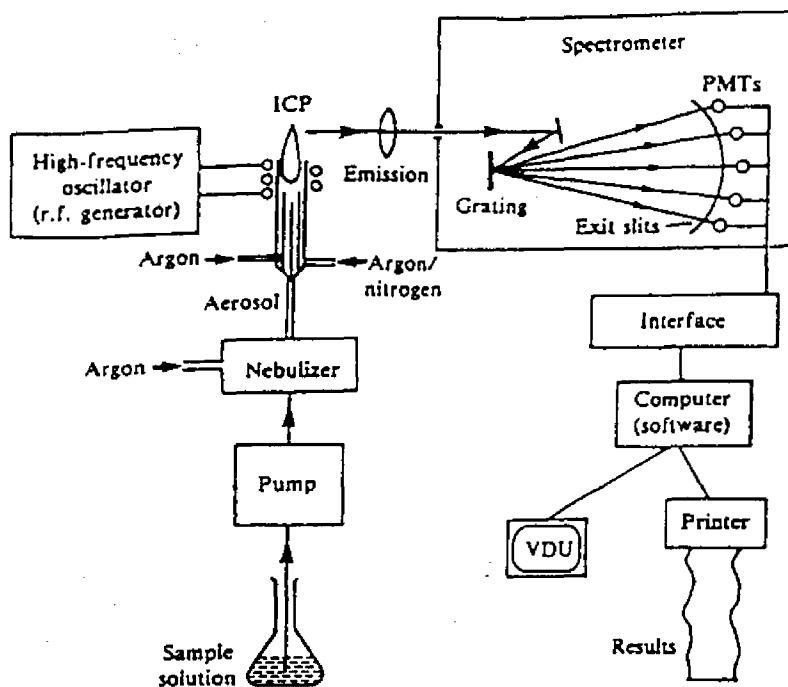
28-10
figure

Schematic of an inductively coupled plasma–atomic emission simultaneous spectrometer. PMT = photomultiplier tube. [From (6), used with permission.]

The innermost tube serves as a sample injection port. The sample is aspirated and nebulized in a fashion similar to that in a flame instrument and is injected into the base of the plasma by the innermost tube.

The extremely high temperatures and the inert atmosphere of argon plasmas are ideal for atomizing and exciting analytes. The absence of oxygen prevents the formation of oxides, which is sometimes a problem with flame methods. The relatively uniform temperature in the plasma (compared to nonuniform temperatures in flames) and the relatively long residence time in the plasma give good linear responses over a several orders of magnitude concentration range.

28.4.4.2 Monochromators, Polychromators, and Echelle Gratings

Sequential PMT instruments are equipped with monochromators that are capable of scanning over a wavelength range, so that readings at several preselected wavelengths can be made rapidly but not simultaneously. In this way, several elements in a single sample can be determined during a single aspiration.

Simultaneous PMT instruments are capable of monitoring several wavelengths simultaneously. These instruments are equipped with polychromators that are preset to separate and focus several spectral lines on a series of photomultiplier tubes arranged around a

semicircle inside the instrument (Fig. 28-12). These instruments have the advantage of being able to analyze several elements very rapidly and with excellent precision. However, they are expensive to buy, and the wavelengths are preset at the factory.

CID instruments are equipped with an eschelle grating. This is a combined prism and diffraction grating in which the emission from the plasma is first directed through a prism and then through a diffraction grating to produce a two-dimensional spectrum that is focused on the CID detector, as further described below.

28.4.4.3 Detectors

As discussed previously, most ICP-AES instruments are equipped with photomultiplier tube detectors. These are excellent detectors but they are capable of measuring the intensity of only one specific wavelength at a time. Another type of detector called a charge injection device (CID) was introduced in 1973 by the General Electric Corporation. It is a solid-state device that contains an array of approximately 95,000 detector elements on a single chip (8). In CID instruments, the emission beam is separated first by a prism and then by a diffraction grating to produce a two-dimensional spectrum that is focused on the CID detector. Thus a quantitative determination of the
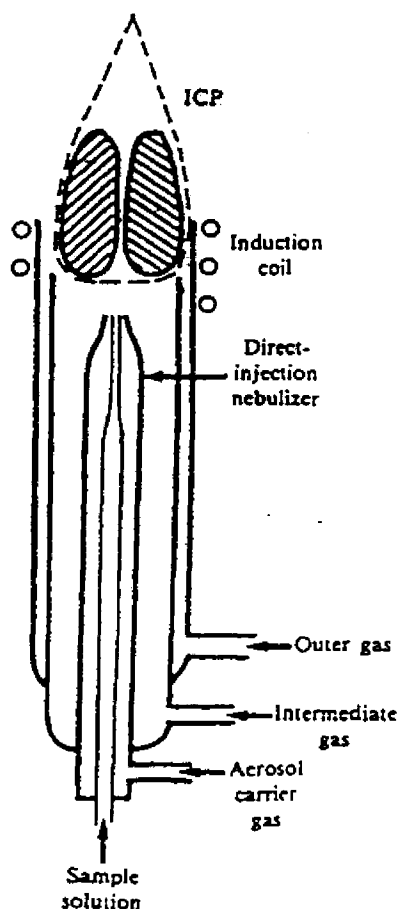
28-11 Schematic of an ICP torch. [From (6), used with permission.]

Labels in figure: ICP / Induction coil / Direct-injection nebulizer / Outer gas / Intermediate gas / Aerosol carrier gas / Sample solution
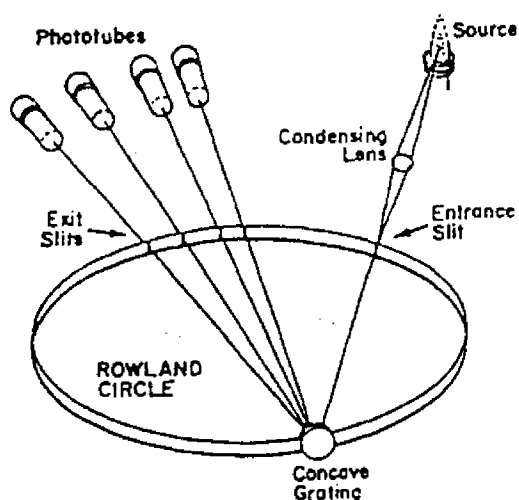
28-12 Schematic of a polychromator for a simultaneous ICP-AES instrument. [From (3), used with permission. Courtesy of the Perkin-Elmer Corporation, Norwalk, CT.]

Labels in figure: Phototubes / Source / Condensing Lens / Exit Slits / Entrance Slit / ROWLAND CIRCLE / Concave Grating

intensity of every spectral line in the emission can be obtained. This is a major advance because multiple elements can be determined simultaneously on a single sample.

28.5 APPLICATIONS OF ATOMIC ABSORPTION AND EMISSION SPECTROSCOPY

28.5.1 Uses

Atomic absorption and emission spectroscopy are widely used for the quantitative measurement of several elements in foods. In principle, any food may be analyzed with any of the atomic spectroscopy methods discussed. In most cases, it is necessary to ash the food to destroy organic matter and to dissolve the ash in a suitable solvent (usually water or dilute HCl) prior to analysis (see Chapter 9 for details on ashing methodology). Proper ashing is critical to accuracy. Some elements may be volatile at temperatures used in dry ashing procedures. Volatilization is less of a problem in wet ashing, but ashing reagents may be contaminated with the analyte. It is therefore wise to carry blanks through the ashing procedure.

Some liquid products may be analyzed without ashing, provided appropriate precautions are taken to avoid interferences. For example, vegetable oils may be analyzed by dissolving the oil in an organic solvent such as acetone or ethanol and aspirating the solution directly into a flame atomic absorption spectrometer. Milk samples may be treated with trichloroacetic acid to precipitate the protein; the resulting supernatant is analyzed directly. A disadvantage of this approach is that the sample is diluted in the process. This may be a problem when analytes are present in low concentrations. An alternative approach is to use a graphite furnace for atomization. For example, an aliquot of an oil may be introduced directly into a graphite furnace for atomization. The choice of method will depend on several factors, including instrument availability, cost, precision/sensitivity, and operator skill.

28.5.2 Practical Considerations

28.5.2.1 Reagents

Since concentrations of many mineral elements in foods are at the trace level, it is essential to use highly pure chemical reagents and water for preparation of samples and standard solutions. Only reagent grade chemicals should be used. Water may be purified by distillation, deionization, or a combination of the two. Reagent blanks should always be carried through the analysis.

28.5.2.2 Standards

Quantitative atomic spectroscopy depends on comparison of the sample measurement with appropriate standards. Ideally, standards should contain the analyte metal in known concentrations in a solution that closely approximates the sample solution in composition and physical properties. A series of standards of varying concentrations should be run to generate a calibration curve. Because many factors can affect the measurement, such as flame temperature, aspiration rate, and the like, it is essential to run standards frequently, preferably right before and/or right after running the sample. Standard solutions may be purchased from commercial sources, or they may be prepared by the analyst. Obviously, standards must be prepared with extreme care the accuracy of the analyte determination depends on the accuracy of the standard. Perhaps the best way to check the accuracy of a given assay procedure is to analyze a reference material of known composition and similar matrix. Standard reference materials may be purchased from the United States National Institute of Standards and Technology (formerly the National Bureau of Standards).

28.5.2.3 Labware

Vessels used for sample preparation and storage must be clean and free of the elements of interest. Plastic containers are preferable because glass has a greater tendency to adsorb metal ions. All labware should be thoroughly washed with a detergent, carefully rinsed with distilled or deionized water, soaked in an acid solution (1 N HCl is sufficient for most applications), and rinsed again with distilled or deionized water.

28.5.3 General Procedure for Atomic Absorption Analysis

While the basic design of all atomic absorption spectrometers is similar, operation procedures do vary from one instrument to another. Therefore, it is always good practice to carefully review operating procedures provided by the manufacturer before using the instrument. Most manuals have detailed procedures for the operation of the instrument as well as tables listing standard conditions (wavelength and slit width requirements, interferences and steps for avoiding them, flame characteristics, linear range, and suggestions for preparing standards) for each element. Be certain to pay close attention to safety precautions recommended by the manufacturer. ACETYLENE IS AN EXPLOSIVE GAS, and great care must be taken to avoid dangerous and damaging explosions.

28.5.3.1 Operation of a Flame Atomic Absorption Instrument

The following is a *generalized procedure* that will be *similar but not identical* to procedures found in instrument operating manuals:

1. Turn the lamp current control knob to the off position.
2. Install the required hollow cathode lamp in the lamp compartment.
3. Turn on main power and power to lamp. Set lamp current to current shown on the lamp label.
4. Select required slit width and wavelength and align light beam with optical system.
5. Ignite flame and adjust oxidant and fuel flow rates.
6. Aspirate distilled water. Aspirate blank and zero instrument.
7. Aspirate standards and sample.
8. Aspirate distilled water.
9. Shut down instrument.

28.5.3.2 Calibration

According to Beer's law, absorbance is directly related to concentration. However, a plot of absorbance versus concentration will deviate from linearity when concentration exceeds a certain level (Fig. 28-13). Therefore, it is always necessary to calibrate the instrument using appropriate standards. This may be done by running a series of standards and plotting absorbance versus concentration or, in the case of most modern instruments, programming the instrument to read in units of concentration.
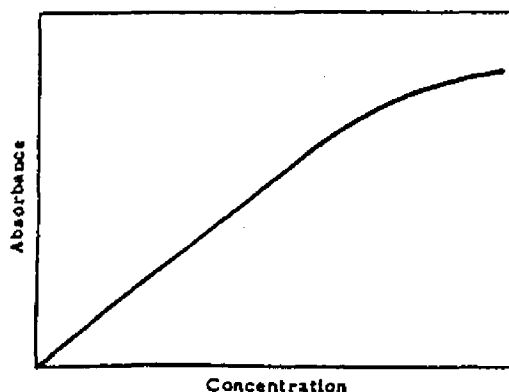
28-13
figure

A plot of absorbance versus concentration showing nonlinearity above a certain concentration. [From (2), used with permission. Courtesy of the Perkin-Elmer Corporation, Norwalk, CT.]

28.5.3.2.1 Selection of Standards The first step in calibration is to select the number and concentrations of standards to use. When operating in the linear range, only one standard is needed. The linear range may be determined by running a series of standards of increasing concentration and plotting absorbance versus concentration. Operating manuals should contain values for linear ranges. The concentration of the standard should be higher than that of the most concentrated sample. If the range of concentration exceeds the linear range, multiple standards must be used. Again, the concentration of the most concentrated standard should exceed the concentration of the most concentrated sample.

28.5.3.2.2 Sensitivity Check Because many factors can influence the operating efficiency of an instrument, it is a good idea to check instrument output using a standard of known concentration. Operating manuals should have values for characteristic concentrations for each element. For example, manuals for Perkin-Elmer atomic absorption spectrophotometers state that a 5.0 mg/liter aqueous solution of iron "will give a reading of *approximately* 0.2 absorbance units." If the measured absorbance reading deviates significantly from this value, appropriate adjustments (e.g., flame characteristics, lamp alignment, etc.) should be made.

28.5.4 General Procedure for ICP-AES

As is the case with atomic absorption spectrometers, operating procedures for atomic emission spectrometers vary somewhat from instrument to instrument. Boss and Fredeen (3) have designed a flow chart that leads the operator through a series of steps to produce a final readout (Fig. 28-14). ICP atomic emission spectrometers are controlled by computers. The software contains methods that specify instrument operating conditions. The computer may be programmed by the operator, or, in some cases, default conditions may be used. Once the method is established, operation is highly automated.

28.6 INTERFERENCES

With any analytical technique, it is important to be on the lookout for possible interferences. Atomic spectroscopy techniques are powerful partly because measurements of individual elements can usually be made without laborious separations. There are two main reasons for this. First, as mentioned previously, a single narrow emission line is used for the measurement. Second, these are relative techniques; that is, quantitative results for an unknown sample are possible only through comparison with a standard of known con-

centration. If there are matrix-effect problems, they can often be overcome by using the same matrix for the standard or by employing the method of additions approach.

28.6.1 Interferences in Atomic Absorption Spectroscopy

The following is a brief discussion of common interference problems in atomic absorption spectroscopy. See references (4) and (6) or your instrument manual for a thorough discussion of interference problems in atomic absorption spectroscopy and reference (5) for a list of interferences for each element. Two types of interferences are encountered in atomic absorption spectroscopy: spectral and nonspectral interference.

28.6.1.1 Spectral Interference

28.6.1.1.1 Absorption of Source Radiation An element in the sample other than the element of interest may absorb at the wavelength of the spectral band being used. Such interference is rare because emission lines from hollow cathode lamps are so narrow that only the element of interest is capable of absorbing the radiation. One example where this problem does occur is with the interference of iron in zinc determinations. Zinc has an emission line at 213.856, which overlaps the iron line at 213.859. The problem may be solved by choosing an alternative emission line for measuring zinc or by narrowing the monochrometer slit width. See reference (4) for a listing of interferences caused by overlapping spectral lines.

28.6.1.1.2 Background Absorption of Source Radiation Particulates present as a result of incomplete atomization may scatter source radiation, thereby attenuating the radiation reaching the detector. This problem may be overcome by going to a higher flame temperature to ensure complete atomization of the sample. Some instruments are equipped with automatic background correction devices. See reference (2) for a description of these devices.

28.6.1.2 Nonspectral Interferences

2.6.1.2.1 Transport Interferences These result when something in the sample solution affects the rate of aspiration, nebulization, or transport into the flame. Transport interferences are rarely a problem with graphite furnace instruments but may cause substantial errors in flame atomic absorption spectroscopy. Such factors as viscosity, surface tension, vapor pressure, and density of the sample solution can influence the rate of transport of sample into the flame. Acid concentration, organic solvents, or dissolved solids may
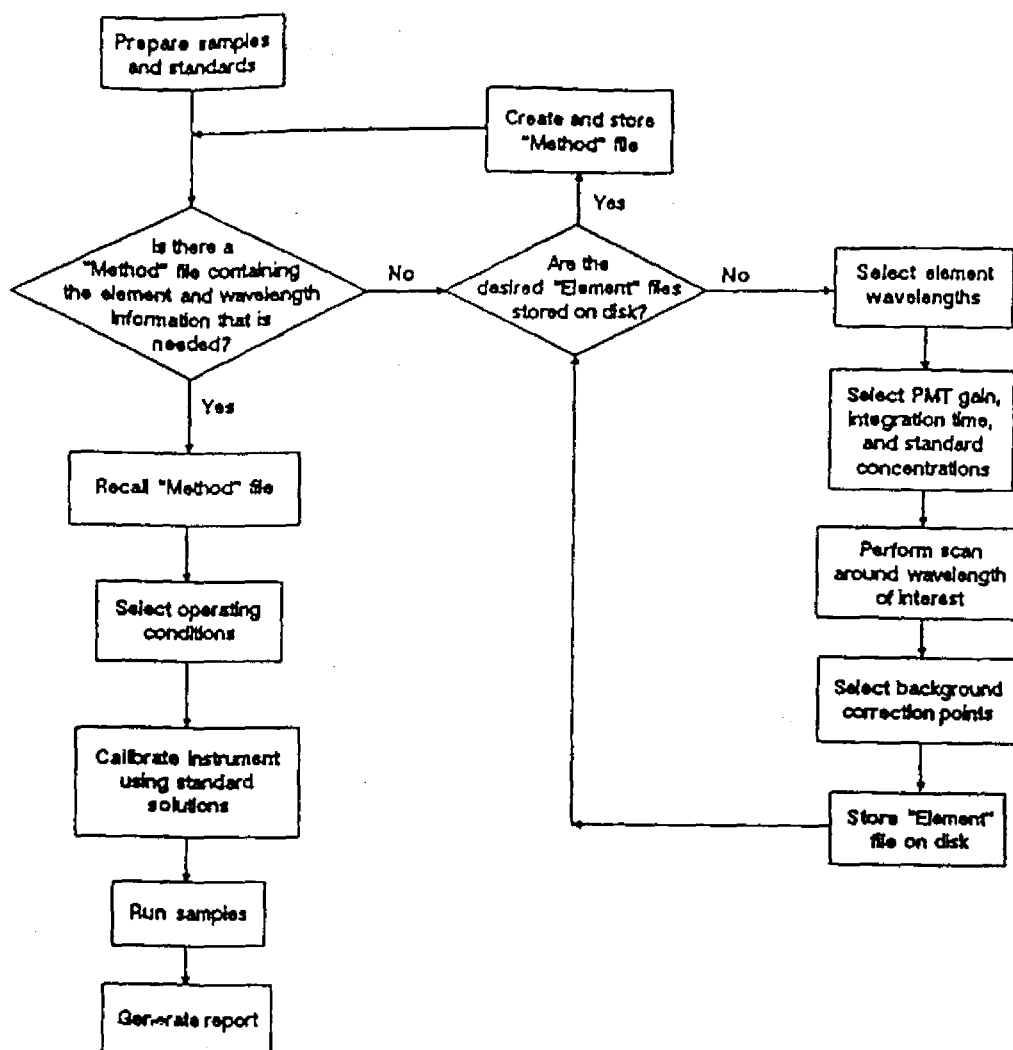
28-14
figure

Steps for operation of an ICP-AES instrument. Once the computer is programmed for a given set of elements, operation is highly automated. [From (3), used with permission. Courtesy of the Perkin-Elmer Corporation, Norwalk, CT.]

affect the absorbance of the analyte. Transport interferences often can be overcome by matching as closely as possible the physical properties of the sample and the standards. For example, use the same solvent for the sample and the reference, or add the interferant in the sample (e.g., sugar) to the standard. The method of additions also may be used to overcome transport interferences.

28.6.1.2.2 Solute Volatilization Interferences These occur when an interferant combines with the element of interest to form a compound of low volatility. This yields a falsely low result because some of the element remains unatomized in the flame. A common example of this is the decrease in calcium absorbance caused by the presence of phosphate in the sample. One approach for overcoming this type of interference is to add

another element, such as lanthanum (as lanthanum oxide), to the sample and reference that will compete with the analyte for compound formation, thereby reducing or eliminating the problem. Another strategy is to use a higher temperature flame; for example, use nitrous oxide–acetylene instead of air–acetylene. A third approach is to add a ligand such as EDTA, which will complex the analyte and prevent it from reacting with the interferant.

28.6.1.2.3 Ionization Interference Ionization of analyte atoms in the flame may cause a significant interference. (Remember that absorption and emission lines of atoms and ions of the same element are different and that atomic absorption spectrometers are tuned to measure *atomic* absorption, not *ionic* absorption. Therefore, any factor that reduces the concentration of atoms

in the flame will lower the absorbance reading). The ionization of atoms results in an equilibrium situation:

$$M \rightleftharpoons M^+ + e^-$$ [5]

Ionization increases with increasing flame temperature and normally is not a problem in air–acetylene flames because the temperature is not high enough. It can be a problem in nitrous oxide–acetylene flames with elements that have ionization potentials of 7.5 eV or less. Ionization is suppressed by the presence of easily ionized elements, such as potassium, through mass action. When potassium ionizes, it increases the concentration of electrons in the flame and shifts the above equilibrium to the left. Reagents added to reduce ionization are called ionization suppressors.

28.6.2 Interferences in ICP-AES

Generally, interferences in ICP-AES analyses are less of a problem than with AAS, but they do exist and must be taken into account. Spectral interferences are the most common. Samples containing high concentrations of certain ions may cause an increase (shift) in background emissions at some wavelengths. This will cause a positive error in the measurement, referred to as background shift interference (see Fig. 28-15). Correction is relatively simple. An emission measurement is made at a wavelength above or below the emission line of the analyte. This emission is then subtracted from the emission of the analyte. Alternatively, another emission line for tungsten in a region where there is no background shift could be chosen.

28.7 COMPARISON OF AAS AND ICP-AES

AAS and ICP-AES have many advantages in common. Both are capable of measuring trace metal concentra-

207.911 nm

5.0 mg/L W

1000 mg/L Al
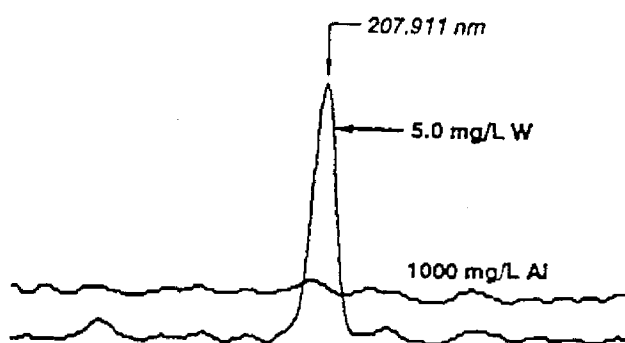
28-15
figure

An example of background shift caused by aluminum is a sample containing tungsten (w). Note that aluminum increases the baseline over a fairly wide wavelength range. [From (3), used with permission. Courtesy of the Perkin-Elmer Corporation, Norwalk, CT.]

tions in complex matrices with excellent precision and accuracy. Sample preparation is relatively simple. For most applications, sample preparation for both techniques involves destruction of organic matter by ashing, followed by dissolution of the ash in an aqueous solvent, usually a dilute acid. In comparison with traditional wet chemistry methods, measurements with AAS and ICP-AES are extremely rapid.

AAS has the advantage of being a more mature technique. There are literally thousands of papers in the literature describing methods for measuring trace element concentrations in hundreds of different matrices. This can be of great value to an analyst because time-consuming methods development can be avoided. Moreover, interferences are well established and relatively easily overcome. Another advantage of AAS is the wide availability of instruments.

ICP-AES instruments are capable of determining concentrations of multiple elements in a single sample with a single aspiration. This offers a significant speed advantage over AAS when the objective is to quantify several elements in a given sample. ICP-AES may also offer an advantage over AAS when analyzing for elements in refractory compounds. Refractory compounds are compounds that are usually stable at high temperatures and may not be fully atomized in the flame of an AAS. Most refractory compounds are readily atomized in the much higher temperatures of a plasma torch.

One way of comparing analytical methods is to compare detection limits. Detection limit has been defined qualitatively as the lowest concentration of the element that can be distinguished from the blank at a given level of confidence. Skoog (reference 5) defines detection limit as follows:

$$\text{Detection limit} = \frac{S_m - S_{bl}}{m}$$ [6]

where:

S_{bl} = the blank signal
S_m = the minimum distinguishable analytical signal; according to Skoog (5)
$S_m = S_{bl} + k s_{bl}$, where s_{bl} is the standard deviation of the blank and k is a constant
m = the slope of the calibration curve at the concentration of interest

Note that the detection limit is a function of the standard deviation of the blank signal and the slope of the calibration curve. The smaller the standard deviation of the blank signal and the steeper the calibration curve, the lower the detection limit. Therefore, detection limit tells us something about the sensitivity (which is a function of m) and the precision of the method.

Table 28-3 lists detection limits for the various

	Flame Atomic	Electrothermal Atomic	Flame	ICP
Element	Absorption	Absorption	Emission	Emission
Al	30	0.005	5	2
Ca	1	0.02	0.1	0.02
Cd	1	0.001	800	2
Cu	2	0.002	10	0.01
Fe	5	0.005	30	0.3
Hg	500	0.01	0.0004	1
Mg	0.01	0.00002	5	0.05
Mn	2	0.0002	5	0.06
Na	2	0.0002	0.1	0.2
Pb	10	0.002	100	2
Zn	2	0.00005	0.0005	2

Table 28-3 Detection Limits (ng/ml) for Selected Elements

Adapted from (5), used with permission of the publishers.
Data from *Principles of Instrumental Analysis*, 3rd ed., by Douglas A. Skoog, copyright ©1985 by Saunders College Publishing, reproduced by permission of the publisher.

methods for elements that may be of interest to food analysts. It should be noted that these are approximate values, and detection limits will vary depending on the sample matrix, the stability of the instrument, and other factors. A two- or threefold difference in detection limit is probably not meaningful, but an order of magnitude difference probably is. Nevertheless, detection limits are useful for choosing among various methods. For example, if you had an atomic spectrometer that could be operated in either the flame atomic absorption mode or the flame emission mode and you wanted to analyze for iron and calcium in spinach, you would probably choose atomic absorption for the iron and atomic emission for the calcium.

28.8 SUMMARY

Atomic absorption spectroscopy quantifies the absorption of electromagnetic radiation by well-separated atoms in the gaseous state, while atomic emission spectroscopy measures emission of radiation from atoms excited by heat or other means. An atomic absorption spectrophotometer uses a hollow cathode lamp as the radiation source and a flame or graphite furnace to atomize the sample. In emission spectroscopy, a flame or plasma (ICP) serves as both the atomizer and excitation source. Development of ICP-AES has revived the use of emission spectroscopy because of its advantages with regard to sensitivity, interferences, and multielement analysis.

Atomic spectroscopy is a powerful tool for the quantitative measurement of elements in foods. The development of this technology over the past 35–40 years has had a major impact on several fields, includ-

ing food science and technology, food safety and toxicology, nutrition, biochemistry, and biology. Today, accurate and precise measurements of a large number of mineral nutrients and non-nutrients in foods can be made rapidly and with minimal sample preparation using commercially available instrumentation.

28.9 STUDY QUESTIONS

1. Explain the significance of energy transitions in atoms and of atomization for the techniques of atomic absorption and atomic emission spectroscopy.
2. Describe the similarities and differences between AAS and AES for mineral analysis.
3. A new employee in your laboratory is somewhat familiar with the application, principles involved, instrumental components, and quantitation procedure for UV-Vis spectroscopy. The employee must now learn to do analyses using AAS. Explain to the new employee the (a) applications, (b) principles, (c) instrumental components and their arrangement (use diagrams and explain differences), and (d) quantification procedure for AAS by comparing and contrasting these same items for UV-Vis spectroscopy. (Assume you are talking about double-beam systems.)
4. What would be the advantages of having an atomic absorption unit that had a graphite furnace?
5. The analytical laboratory in your company plans to purchase an inductively coupled plasma–atomic emission spectrometer.
   a. Explain the instrumentation and principle of its operation to analyze foods for specific minerals.
   b. Explain how AAS differs in instrumentation and principle of operation from what you described previously for ICP-AES.
   c. What are the advantages of ICP-AES over AAS?
   d. The analytical lab in your company handles a large number of samples and analyzes them for multiple elements. Would you request purchase of a simultaneous photomultiplier tube (PMT) spectrometer, a sequential PMT spectrometer, or a charge injection device (CID) spectrometer? Explain your answer.
   e. For most types of food samples other than clear liquids, what type of sample preparation and treatment is generally required before using ICP-AES, AES, or AAS for analysis?
6. In your preparation of an ashed milk sample for calcium determination by atomic absorption, you forgot to add either EDTA or LaCl₃. Would you likely over- or underestimate the true Ca content? Why would it likely be necessary to add one of these to obtain accurate results? Briefly explain how each of these works.
7. In the quantitation of Na by atomic absorption, KCl or LiCl was not added to the sample. Would you likely over- or underestimate the true NA content? Explain why either KCl or LiCl is necessary to obtain accurate results.
8. Give five potential sources of error in sample preparation prior to atomic absorption analysis.
9. The detection limit for calcium is lower for ICP emission

than it is for flame atomic absorption. How is the detection limit determined, and what does it mean?

10. As the manager of the quality assurance laboratory for your company, you ask one of your technicians to find the AOAC methods for sodium determination in a specific food product. Your technician finds the following methods listed: Volhard titration, ion selective electrode, and ICP-AES. Your technician asks you about the differences between these methods. To answer the question, differentiate the principles involved, and explain why your lab might choose to use one method over the other. (See also Chapter 10.)

11. Calibration curves (i.e., standard curves) are used in (a) ultraviolet-visible spectroscopy, (b) ion-selective electrodes, and (c) atomic emission spectroscopy. For each method, state what factors are plotted against each other, and state what type of curve is expected (i.e., linear or nonlinear, positive or negative slope). (See also Chapters 10 and 26.)

28.10 PRACTICE PROBLEMS

1. The following data were recorded during a procedure for determining the iron content of enriched flour. Calculate the iron concentration in the flour. Express your answer as mg Fe/lb flour. The protocol was as follows:

Weigh out 10.00 g of the flour. Transfer to a 800-ml Kjeldahl flask. Add 20 ml of H_2O, 5 ml of H_2SO_4, and 25 ml of HNO_3. Heat to SO_3 fumes. Cool, add 25 ml of H_2O, filter quantitatively into a 100-ml vol. flask. Dilute to volume. Prepare iron standards with concentrations of 0, 2, 5 mg Fe/liter. Read absorbances of standards and sample on an atomic absorption spectrophotometer.

Sample	Fe Concentration (mg/liter)	Absorbance	Corrected Absorbance
Std 1	0.00	0.01	0.00
Std 2	2.0	0.21	0.20
Std 3	5.0	0.51	0.50
Flour Fe	?	0.38	0.37

2. Describe a procedure for determining calcium, potassium, and sodium in infant formula using an ICP-AES. Note: Concentrations of Ca, K, and Na in infant formula are around 700, 730, and 300 mg, respectively.

Answers

1. 18.16 mg Fe/lb flour
2. Consult AOAC International. 1995. Official Methods of

Analysis, 16th ed., AOAC Method 984.27, Locator No. 50.1.15. The following approach may be used:

a. Shake can vigorously.
b. Transfer 15.0 ml of formula to a 100-ml Kjeldahl flask. (Carry two reagent blanks through with sample.)
c. Add 30 ml of HNO_3-$HClO_4$ (2:1).
d. Leave samples overnight.
e. Heat until ashing is complete (follow AOAC procedure carefully—mixture is potentially explosive.)
f. Transfer quantitatively to a 50-ml vol. flask. Dilute to volume.
g. Calibrate instrument. Choose wavelengths of 317.9 nm, 766.5 nm, and 589.0 nm for Ca, K, and Na, respectively. Prepare calibration standards containing 200, 200, and 100 ng/ml for Ca, K, and Na, respectively.
h. The ICP-AES computer will calculate concentrations in the samples as analyzed. To convert to concentrations in the formula, use the following equation:

$$\text{Concentration in formula} = \text{Concentration measured by ICP} \times \frac{50 \text{ ml}}{15 \text{ ml}}$$

28.11 REFERENCES

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29
chapter

Mass Spectrometry

J. Scott Smith and Rohan A. Thakur

29.1 INTRODUCTION

Mass spectrometry is unique among the various spectroscopy techniques in both theory and instrumentation. As you may recall, spectroscopy involves the interaction of electromagnetic radiation or some form of energy with molecules. The molecules absorb the radiation and produce a spectrum either during the absorption process or as the excited molecules return to the ground state. Mass spectrometry works by placing a charge on a molecule, thereby converting it to an ion in a process called ionization. The generated ions are then resolved according to their mass-to-charge ratio (m/z) by subjecting them to electrostatic fields (mass analyzer), and finally detected. An additional stage of ion fragmentation may be included before detection to elicit structural information in a technique known as tandem MS. The result of ion generation, separation, fragmentation, and detection is manifested as a mass spectrum that can be interpreted to yield molecular weight or structural information. The uniqueness of this process allows the method to be used for both detection and identification of an unknown compound.

Because of recent advances in instrument design, electronics, and computers, mass spectrometry has become routine in many analytical labs. Probably the most common application is the interfacing of a mass spectrometer with gas chromatography (GC) (see Chapter 33), in which the mass spectrometer is used to confirm the identity of compounds as they elute off the GC column. The use of high performance liquid chromatography (HPLC) (see Chapter 32) with the mass spectrometer also has recently become more routine due to advances made in the interconnecting interfaces.

29.2 INSTRUMENTATION—THE MASS SPECTROMETER

29.2.1 Overview

The mass spectrometer (MS) performs three basic functions. There must be a way to ionize the molecules, which occurs in the ion source by electron ionization, fast atom/ion bombardment, matrix-assisted-laser-desorption, or electrospray. The charged molecular ion and its fragments must be separated according to their m/z, and this occurs in the mass analyzer section (e.g., quadrupoles, ion traps, time-of-flight). Finally the separated, charged fragments must be monitored by a detector. The block diagram in Fig. 29-1 represents the various components of a mass spectrometer.

Sample introduction can be static, direct insertion probes, or dynamic, which involves interfacing chromatographic equipment such as gas or liquid chromatography. Interface methods include heated capillary transfer lines (GC–MS), and LC–MS techniques such as thermospray, electrospray, and atmospheric pressure ionization interfaces. Common mass spectrometer interfaces will be discussed in more detail in the sections on GC–MS and LC–MS.

Figure 29-2 depicts the interior of a typical quadrupole mass spectrometer. The region between ion generation and detection is maintained by different vacuum pumps. Each successive region from the source is kept at lower vacuum than the preceding region, with the mass analyzer/detector being in the region of strongest vacuum ($\sim 10^{-5}$ Torr). A vacuum is necessary to avoid ion–molecule reactions between the charged ions and other gaseous molecules before they reach the detector, thereby increasing both sensitivity and resolution.

29.2.2 Sample Introduction

The initial step in operating the MS is to get the sample into the ion source chamber. With pure compounds, that are a gas or a volatile liquid, it is injected directly into the source region. This requires no special equipment or apparatus and is much the same as injecting a sample into a GC. Thus, this static method of introducing the sample to the source is called direct injection. With solids that are at least somewhat volatile, the direct insertion probe method is used, in which the sample is placed in a small cup at the end of a stainless steel rod or probe. The probe is inserted into the ion source through one of the sample inlets, and the source is heated until the solid vaporizes. The mass spectrum is then obtained on the vaporized solid material as with the direct injection method.

Both direct injection and direct insertion probe methods work well with pure samples, but their use is very limited when analyzing complex mixtures of several compounds. For mixtures, sample introduction is a dynamic method in which the sample must be separated into the individual compounds and then analyzed by the mass spectrometer. This is done typically by GC or HPLC connected to a mass spectrometer by an interface (see sections 29.4 and 29.5). The interface removes excess GC carrier gas or HPLC solvent that would otherwise overwhelm the vacuum pumps of the MS.

29.2.3 Ionization

In GC-MS analysis, once in the ion source, the compound is exposed to a beam of electrons emitted from a filament composed of rhenium or tungsten metal. When a direct current is applied to the filament (usually 70 electron volts), it heats and emits electrons that
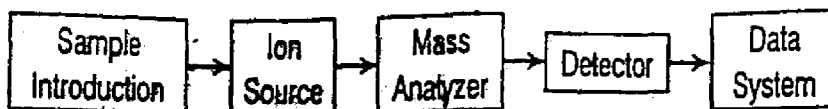
29-1 figure A block diagram of the major components of a mass spectrometer.
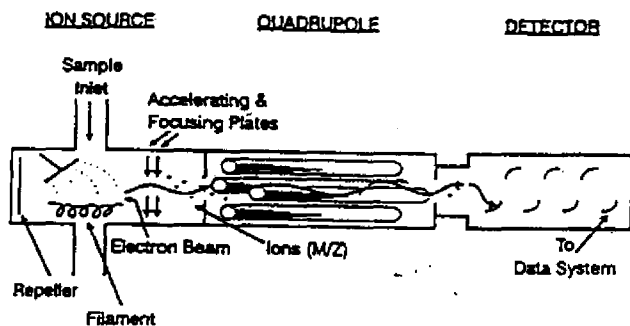
29-2 figure Schematic of a typical mass spectrometer. The sample inlets (interfaces) at the top and bottom can be used for direct injection or interfacing to a GC.

move across the ion chamber toward a positive electrode on the other side. As the electrons pass through the source region, they come in close proximity to the sample molecule and extract an electron, forming an ionized molecule. Once ionized, the molecules contain such high internal energies they can further fragment into smaller molecular fragments. This entire process is called electron impact (EI) ionization, although the emitted electrons rarely hit a molecule.

The eventual outcome of the ionization process is both negatively and positively charged molecules of various sizes unique to each compound. When the repeller plate at the back of the source is positively charged, it repels the positive fragments toward the quadrupole mass analyzer. Thus, we look only at the positive fragments, although negative fragments are sometimes analyzed. As the positively charged fragments leave the ion source, they pass through holes in the accelerating and focusing plates. These plates serve to increase the energy of the charged molecules and to focus the beam of ions, so that a maximum amount reaches the mass analyzer.

29.2.4 Mass Analyzers

The heart of a mass spectrometer is the mass analyzer. It performs the fundamental task of separating the charged fragments based on their m/z, and dictates the mass range, accuracy, and sensitivity. There are five common types of mass analyzers: quadrupoles, ion traps, time-of-flight (TOF), magnetic sectors, and

Fourier-transform-ion cyclotrons (FT-ICR). Combinations of these basic mass analyzer types greatly enhance the information derived from routine mass spectrometry. Over the years, quadrupole-MS has become increasingly popular; however, recent improvements in ion trap-MS (both in cost and ease-of-use) have brought the advantages of multiple stages of mass spectrometry (MS") to the routine user.

Magnetic sector analyzers have been used in mass spectrometers for years and continue to be very popular, especially in high-resolution instruments. Because of the large size of the magnet sector, analyzers are not commonly used in bench-top spectrometers and largely have been replaced by the quadrupole mass filter.

As the name implies, the magnetic sector analyzer uses a magnetic field to separate the ions based on their m/z. As shown in Fig. 29-3 ions produced in the ion source region of the instrument are accelerated down a curved tube that runs through a magnet. Through a combination of magnetic field strength and ion velocity, the ions take on a curved path to the detector. Ions having a curved path that keeps them in the center of the analyzer tube will reach the detector opening. Ions that hit the sides of the tube are pumped away and do not reach the detector.

To detect multiple ions, to produce the typical mass spectra, the magnet field strength is changed so that all possible m/z ratios are seen. If the field strength is changed very quickly, then all of the ions are detected almost instantaneous.
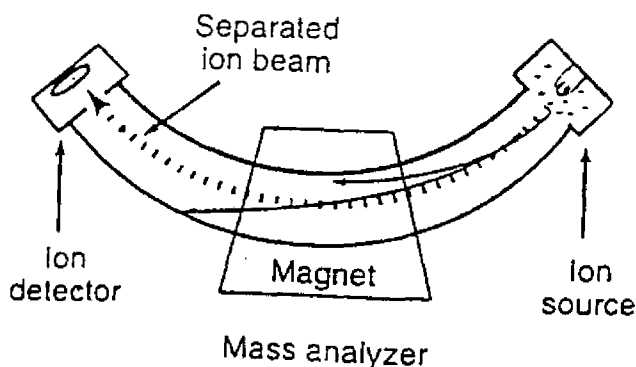
Mass analyzer

29-3 figure Diagram of a magnetic sector mass analyzer.

Quadrupole mass analyzers are based on the ion-focusing work of the Greek electrical engineer Christophilos. The word "quadrupole" is derived from the Latin word for four (quadruplus), and "pole," to describe the array of four rods that are used. The four rods are used to generate two equal but out-of-phase electric potentials; one is alternating current (AC) frequency of applied voltage that falls in radiofrequency (RF) range, and one is direct current (DC). The potential difference can be varied to create an oscillating electrical field between two of the opposite rods, resulting in their having equal but opposite charges.

When, for example, a positive-charged ion enters the quadrupole field it will be instantly attracted toward a rod maintained at a negative potential, and if the potential of that rod changes before the ion impacts, it will be deflected, i.e., change direction. Thus, every stable ion entering the quadrupolar region traces a sine-wave-type pattern on its way to the detector. By adjusting the potentials on the rods, selected ions, a mass range, or only a single ion can be made stable and detected. The unstable ions impact one of the four rods, releasing them from the influence of the oscillating field, and are pumped away by the vacuum pumps.

Ion traps are essentially three-dimensional quadrupole mass analyzers that store ions (trap) and then eject these trapped ions according to their m/z ratios. Once the ions are trapped, multiple stages of mass spectrometry (MS^n) can be achieved, mass resolution can be increased, and sensitivity can be improved. The major difference between an ion trap and a quadrupole mass analyzer is that in an ion trap the unstable ions are ejected and detected while the stable ions are trapped, whereas in a quadrupole, the stable ions reach the detector, and the unstable ions hit the rods and are pumped away.

Figure 29-4 shows the cross-sectional view of the ion trap mass analyzer. It consists of a ring electrode sandwiched between a perforated-entrance, end-cap electrode and a perforated-exit, end-cap electrode. An AC (RF) voltage and variable amplitude is applied to the ring electrode, producing a three-dimensional quadrupole field within the mass analyzer cavity.

Ions formed in the source are electronically injected into the ion trap, where they come under the influence of a time-varying RF field. The ions are trapped within the mass analyzer cavity, and the applied RF voltage drives ion motion in a figure of eight toward the end-caps. Thus, for an ion to be trapped it must have a stable trajectory in both the axial and radial directions. To detect the ions, the frequency applied to the ring electrode is changed and the ion trajectories are made unstable. This results in the ions being ejected through the perforated end-caps, thus enabling them to impinge on the detector.

Ions in
End-cap electrode
Oscillating
Ions trapped
Ring electrode
Ions to detector
End-cap electrode
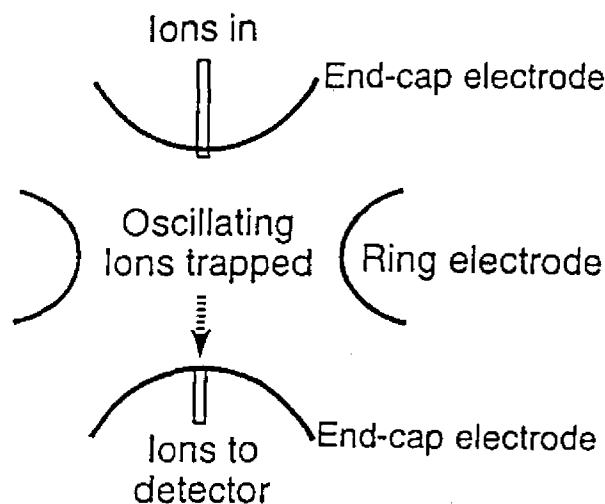
29-4 Diagram of an ion trap mass analyzer.

Helium is continuously infused into the ion trap cavity, and primarily serves as a dampening gas. Being lighter than any ions entering the trap (low mass cutoff is normally m/z 50), it nonelastically collides with the ions entering the trap. This results in the helium molecules (not ions) absorbing kinetic energy from the ions entering the trap, effectively dampening and focusing the ions to the center of the trap, where they can be trapped more efficiently. This process also improves sensitivity and resolution in the trap.

29.3 INTERPRETATION OF MASS SPECTRA

As previously indicated, a mass spectrum is a plot (or table) of the intensity of various mass fragments (m/z) produced when a molecule is subjected to one of the many types of ionization techniques. With classical GC-MS the electron beam generated by a heated filament is used to ionize the molecules. It is usually kept at a constant potential of 70 electron volts because this produces sufficient ions without too much fragmentation, which would result in a loss of the higher molecular weight ions. Another advantage of using 70 electron volts for ionization is that the resulting mass spectra are usually very similar regardless of the make and model of the instrument. This allows for computer-assisted mass spectral matching to data base libraries that help in unknown compound identification. In fact, most mass spectrometers now come with a MS spectral data base and the required matching software.

Typical mass spectra include only positive fragments that usually have a charge of +1. Thus, the mass-to-charge ratio is the molecular mass of the fragment

divided by +1, which equals the mass of the fragment. As yet, the mass-to-charge ratio unit has no name and is currently abbreviated by the symbol m/z (older books use m/e). In some publications the unit of mass-to-charge ratio is called the Thomson after the late J.J. Thomson, who constructed one of the first instruments for the determination of the m/z of ions.

A mass spectrum for butane is illustrated in Fig. 29-5. The relative abundance is plotted on the y-axis and the m/z is plotted on the x-axis. Each line on the bar graph represents an m/z fragment with the abundance unique to a specific compound. The spectrum always contains what is called the base peak or base ion. This is the fragment (m/z) that has the highest abundance or intensity. When the signal detector is processed by the computer, the m/z with the highest intensity is taken to be 100%, and the abundance of all the other m/z ions is adjusted relative to the base peak. The base peak always will be presented as 100% relative abundance. Butane has the base peak at an m/z of 43.

Another important fragment is the molecular ion or parent ion, designated by the symbol M^+. This peak has the highest mass number and represents the positively charged intact molecule with an m/z equal to the molecular mass. The harsher ionizing techniques such as the electron impact (EI) shown here produces an ion (radical cation) by stripping an electron. Because the mass of a single electron can be considered insignificant, the molecular ion produced by EI type ionization is indicative of the molecular weight of that compound. All other molecular fragments originate from this charged species, so it is easy to see why it is called the molecular or parent ion. It is not always present because sometimes the parent ion decomposes before it has a chance to traverse the mass analyzer. However, a mass spectrum is still obtained, and this becomes a problem only when determining the molecular mass of an unknown. The remainder of the mass spectrum is a consequence of the stepwise cleavage of large fragments to yield smaller ones termed product ions or

daughter ions. The process is relatively straightforward for alkanes, such as butane, making identification of many of the fragments possible.

As indicated previously, the initial step in electron impact ionization is the abstraction of an electron from the molecule as electrons from the beam pass in close proximity. The equation below illustrates the first reaction that produces the positively charged product ion.

M + e
(from electron beam) ⟹

M^+ (molecular ion)
+
2e (one electron from the electron beam and one from the molecular ion, M) [1]
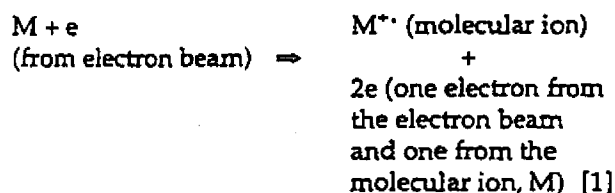
The M symbolizes the un-ionized molecule as it reacts with the electron beam and forms a radical cation. The cation will have an m/z equal to the molecular weight. The parent ion then sequentially fragments in an unimolecular fashion. (Note that the product ion is often written as M^+, for which the free electron, symbolized by the dot, is assumed. Regardless, the molecule has lost one electron and still retains all the protons; thus, the net charge must be positive.) The reactions of butane as it forms several of the predominant product ion (daughter) fragments are shown below.

$CH_3-CH_2-CH_2-CH_3 + e \Rightarrow CH_2-CH_2-CH_2-CH_3^+$
$(m/z = 58) + 2e$ [2]

$CH_3-CH_2-CH_2-CH_3^+ \Rightarrow CH_3-CH_2-CH_2-CH_2^+$
$(m/z = 57) + \cdot H$ [3]

$CH_3-CH_2-CH_2-CH_3^+ \Rightarrow CH_3-CH_2-CH_2^-$
$(m/z = 43) + \cdot CH_3$ [4]

$CH_3-CH_2-CH_2-CH_3^+ \Rightarrow CH_3-CH_2^+$
$(m/z = 29) + \cdot CH_3-CH_3$ [5]

$CH_3-CH_2 \Rightarrow CH_3^+ (m/z = 15) + \cdot CH_2$ [6]
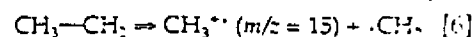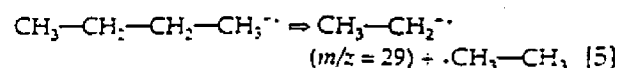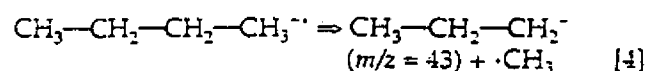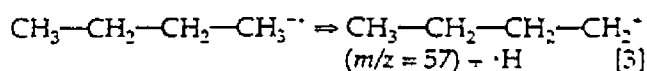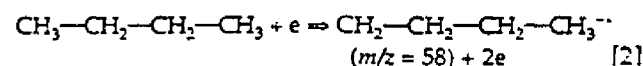
Many of the fragments for butane result from direct cleavage of the methylene groups. With alkanes, you will always see fragments in the mass spectrum that are produced by the sequential loss of CH_2 or CH_3 groups.

Close examination of the butane mass spectrum in Fig. 29-5 reveals a peak that is 1 m/z unit larger than the molecular ion at m/z = 58. This peak is designated by the symbol M + 1 and is due to the naturally occurring isotopes. The most abundant isotope of carbon has a mass of 12; however, a small amount of ^{13}C is also present (1.11%). Any ions that contained a ^{13}C or a deuterium isotope would be 1 m/z unit larger, although the relative abundance would be low.

Another example of MS fragmentation patterns is shown for methanol in Fig. 29-6. Again, the fragmenta-

$CH_3 \cdot CH_2 \cdot CH_2 \cdot CH_3$
Mol Wt = 58

43
Base Peak

Parent Ion
58

15   29

MASS/CHARGE

RELATIVE ABUNDANCE
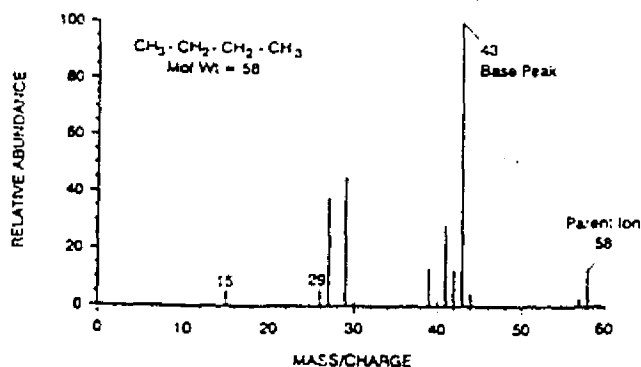
Mass spectrum of butane obtained by electron impact ionization.
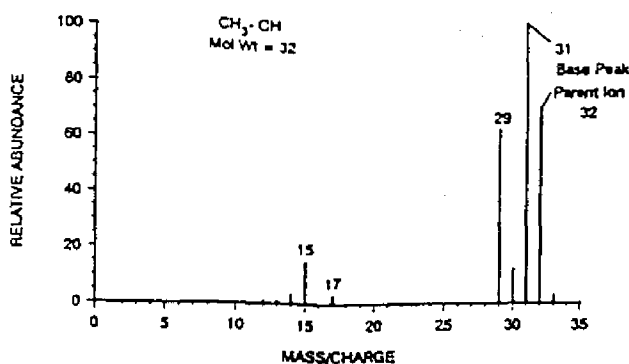
29-6 figure Mass spectrum of methanol obtained by electron impact ionization.

tion pattern is straightforward. The molecular ion (CH_3—$OH^{+\cdot}$) is at an m/z of 32, which is the molecular weight. Other fragments include the base peak at an m/z of 31 due to CH_2—OH^+, the CHO^+ fragment at an m/z of 29, and the CH_3^+ fragment at an m/z of 15.

So far, only EI types of ionization have been discussed. Another common fragmentation method is chemical ionization (CI). In this technique, a gas is ionized, such as methane (CH_4), which then directly ionizes the molecule. This method is classified as a soft ionization because only a few fragments are produced. The most important use of CI is in the determination of the molecular ion since there is usually a fragment that is 1 m/z unit larger than that obtained with EI. Thus, a mass spectrum of butane taken by the CI method would have a quasimolecular (parent) ion at m/z = 59 (M + H). As can be seen, the reactions of the cleavage process can be quite involved. Many of the reactions are covered in detail in the books by McLafferty and Turecek and by Davis and Frearson listed in the resource materials section.

29.4 GAS CHROMATOGRAPHY–MASS SPECTROMETRY

Although samples can be introduced directly into the mass spectrometer ion source, many applications require separation before analysis. The rapid development of gas chromatography–mass spectrometry (GS–MS) has allowed for the coupling of the two methods for routine separation problems. As discussed in Chapter 33, gas chromatography is a powerful separation method applicable to many different types of food compounds. However, one of the problems with GC analysis is identifying the many compounds that elute off the column. In some cases, there may be GC peaks present that are unknown. A mass spectrometer coupled to GC allows the peaks to be identified or confirmed, and, if an unknown is present, it can be identi-

fied using a computer-assisted search of a library containing known MS spectra. Another critical function of GC–MS is to ascertain the purity of each peak as it elutes from the column. Does the material eluting in a peak contain one compound, or is it a mixture of several that just happen to coelute with the same retention time?

Connecting a GC to a mass spectrometer is straightforward and requires one additional component, the interface. Since a GC operates with a carrier gas, there must be some way to remove or minimize the large amounts of gas going into the MS so that the vacuum is not compromised.

Figure 29-7 shows a diagram of a jet-separator type of interface. As the carrier gas and compounds pass through the interface, the small gas molecules are pumped away, while the compounds of interest move in a straight path toward the ion source.

Since the advent of capillary GC columns, the interfaces have become much simpler, with many consisting of only a heated region where both the gas and compounds to be analyzed go directly into the MS source (i.e., heated capillary transfer lines). The direct interface is possible because capillary columns require considerably less gas flow. A cutaway view of the interior of a GC–MS is shown in Fig. 29-8. As you can see, the sample flows through the GC column into the interface and then on to be processed by the MS. A computer is used to store and process the data from the MS.

One other modification required for GC–MS is a detector for the ions as they leave the source region. A small ion collector plate is placed at the end of the source and monitors the total ions or total ion current as they go into the quadrupoles. This allows a total-ion GC chromatogram to be recorded in addition to the mass spectra.

An example of the power of GC–MS is shown below in the separation of the methyl esters of several long-chain fatty acids (Fig. 29-9). Long-chain fatty
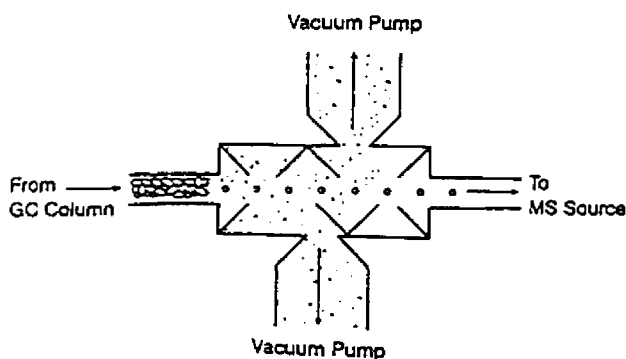
29-7 figure Jet separator interface for the GC–MS. The small dots represent carrier gas, and the open circles are the compounds eluting off the columns.
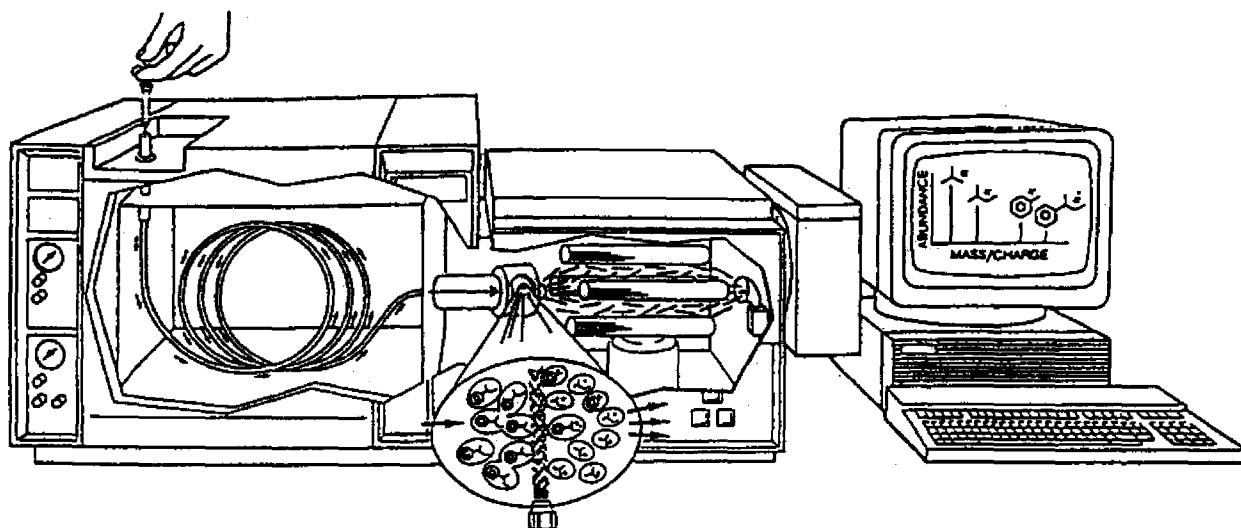
29-8 figure Diagram of a gas chromatograph connected to a small tabletop mass selective detector. (Courtesy of Hewlett-Packard Co., Avondale, PA.)

acids must have the carboxylic acid group converted or blocked with a methyl group to make them volatile. Methyl esters of palmitic (16:0), oleic (18:1), linoleic (18:2), linolenic (18:3), stearic (18:0), and arachidic (20:0) acids were injected onto a column that was supposed to be able to separate all the naturally occurring fatty acids. However, the GC tracing showed only four peaks, when it was known that six different methyl esters were in the sample. The logical explanation is that several of the peaks contain a mixture of methyl esters resulting from poor resolution on the GC column.

The purity of the peaks is determined by running the GC–MS and taking mass spectra at very short increments of time (1 sec or less). If a peak is pure, then the mass spectra taken throughout the peak should be the same. In addition, the mass spectrum can be compared with the library of spectra stored in the computer.

The total ion current (TIC) chromatogram of the separation of the fatty acid methyl esters is shown in Fig. 29-9. There are four peaks eluting off the column between 15.5 min and 28 min. The first peak at 15.5 min has the same mass spectrum throughout, indicating that only one compound is eluting. A computer search of the MS library gives an identification of the peak to the methyl ester of palmitic acid. The mass spectra shown in Figure 29-10 compare the material eluting from the column to the library mass spectrum.

Most of the fragments match, although the GC–MS scan does have many small fragments not present on the library mass spectrum. This is common background noise and usually does not present a problem.
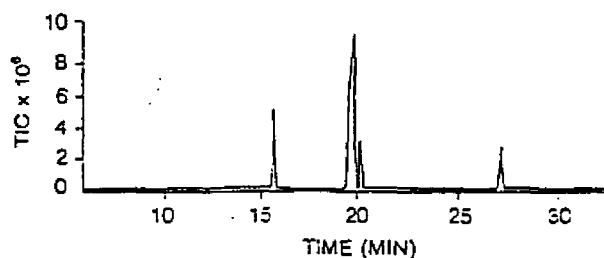
29-9 figure Total ion current (TIC) GC chromatogram of the separation of the methyl esters of six fatty acids. Detection is by electron impact (EI) ionization using a direct capillary interface.

The data from the rest of the chromatogram indicate that the peaks at both 20 min and 27 min contain only one component. The computer match identifies the peak at 20 min as stearic acid, methyl ester, and the peak at 27 min as arachidic acid, methyl ester. However, the peak located at 19.5 min is shown to have several different mass spectra, indicating impurity or coeluting compounds.

In Fig. 29-11, the region around 19 min has been enlarged. The arrows indicate where different mass spectra were obtained. The computer identified the material in the peak at 19.5 min as linoleic acid, methyl ester; the material at 19.7 min as oleic acid, methyl ester; and the material at 19.8 min as linolenic acid, methyl ester. Thus, as we originally suspected, several of the methyl esters were coeluting off the GC column. This example illustrates the tremendous power of GC–MS used in both a quantitative and a qualitative manner.
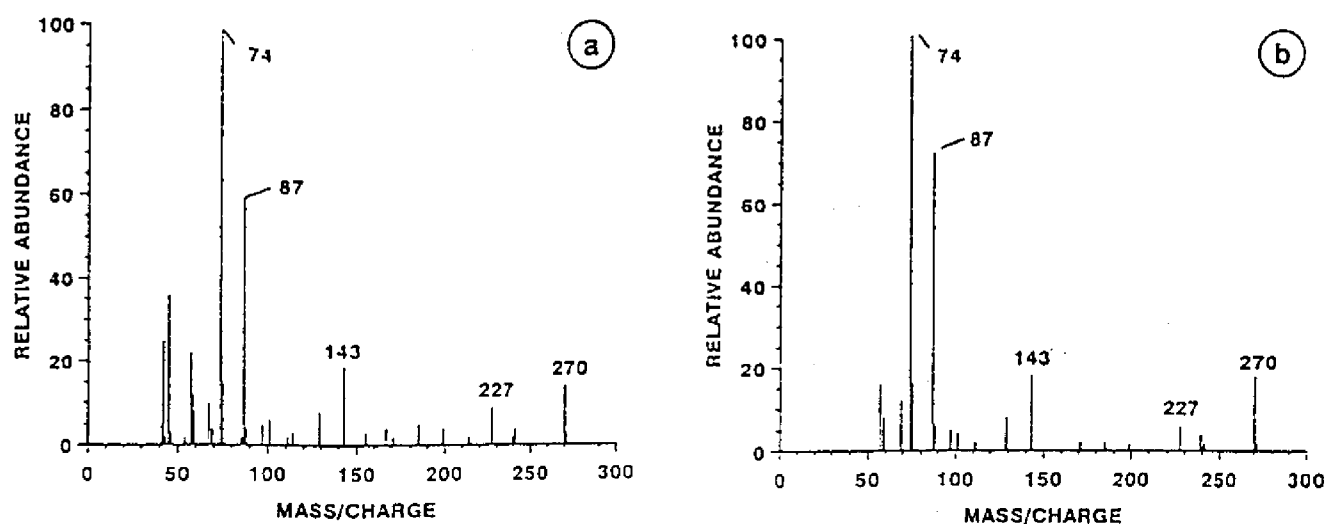
29-10 figure Mass spectra of (a) the peak at 15.5 min in the TIC chromatogram shown in Fig. 29-9 and (b) the methyl ester of palmitic acid from a computerized MS library.
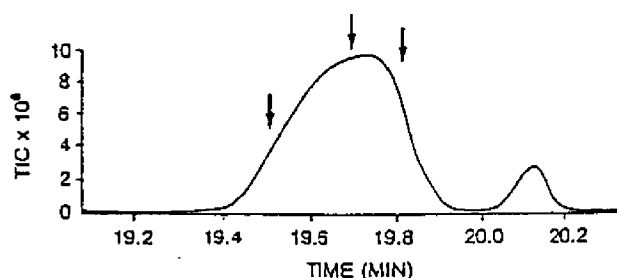
29-11 figure Enlargement of the region 19.2–20.2 min from the TIC chromatogram shown in Fig. 29-9. Arrows indicate where mass spectra were obtained.

29.5 LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

Years ago the only way to obtain a decent mass spectrum of material from high performance liquid chromatography (HPLC) separations was to collect fractions, evaporate off the solvent, and introduce the sample into a conventional MS by direct injection or direct probe. Although this method was sometimes adequate, the direct on-line coupling of the two instruments was a tremendous advantage in terms of time and ease of operation.

For a high performance liquid chromatography–mass spectrometry (LC–MS) interface, the same overall requirements must be met as for GC–MS. There must be a way to remove the excess solvent, while converting a fraction of the liquid effluent into the gas phase, making it amenable for mass spectrometry analysis. Furthermore most compounds analyzed by

HPLC are either nonvolatile or thermally labile, making the task of liquid-to-gas phase transition even more challenging, especially while maintaining compound integrity.

How does LC–MS work? Remember that evaporation is a cooling process, especially when performed under conditions of reduced pressure (adiabatic expansion) and increased volume. A basic LC–MS interface facilitates desolvation by application of heat, followed by a rapid expansion of this vapor in an area of reduced pressure. Thus the heat energy applied to evaporate the solvent is completely used in the desolvation process, and does not contribute to degradation of any thermally labile species present in the LC eluant. There are many different types of interfaces though several have come to the forefront of use in recent years. Three of the most commonly used interfaces are the thermospray (TSP), electrospray (ESI), and atmospheric pressure chemical ionization (APCI).

29.5.1 Thermospray Interface

The thermospray interface, as its name suggests, removes heated solvent as it is sprayed into the ion source. A simple diagram of the thermospray interface is shown in Fig. 29-12. As the effluent leaves the HPLC instrument, it is pumped through a small piece of tubing into the thermospray probe. The effluent moves along the heated probe and exits at the tip as a spray. The solvent is removed from the spray by several vacuum pumps as small particles of sample plus solvent travel through the ion source to be ionized. The ionization process is considerably different from what occurs with GC–MS in that very little fragmentation occurs.
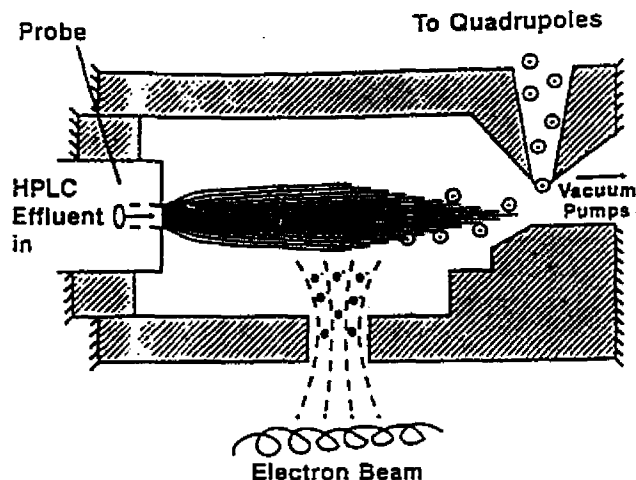
Schematic of a thermospray LC–MS interface. (Courtesy of Hewlett-Packard Co., Avondale, PA.)

An important aspect of thermospray–MS (TS–MS) is that some type of volatile buffer must usually be present when the HPLC effluent exits the tip. The ionization is a reaction between the volatile buffer and the compounds as the solvent evaporates from the droplets in the source region. Ammonium acetate is commonly used; it produces ammonium ions that react in several ways. The ammonium ions can provide either H^+ or NH_4^+ to the molecule, which yields an $M + H^+$ molecular ion or an $M + NH_4^+$ molecular ion. In any case, the compounds do not fragment much, producing a very simple mass spectrum. The electron beam filament shown in Fig. 29-12 may be used to enhance the ionization and increase the production of ions, although it is not necessary for many compounds.

29.5.2 Electrospray Interface (ESI)

Electrospray, the most popular LC–MS technique in use today, was developed by Fenn and co-workers in 1984. It is a very sensitive technique with a limit of sensitivity normally in the femtogram range. Normally, polar compounds are amenable to ESI analysis with the type of ion produced depending on the initial charge. That is, positively charged compounds yield positive ions, while negatively charged compounds such as those containing free carboxylic acid functional groups will produce negative ions.

The ESI source as depicted in Figure 29-13 consists of a nozzle that contains a fused-silica capillary sample tube (serves to transfer the LC effluent) coaxially positioned within a metal capillary tube to which a variable potential can be applied. Nitrogen is normally coaxially infused to aid in the nebulization of the LC effluent
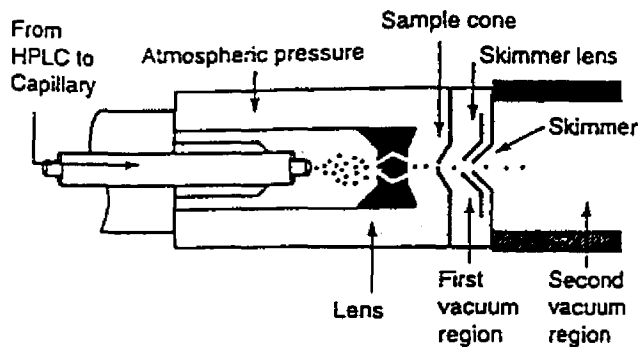
Schematic of an electrospray LC–MS interface. (Courtesy of Finnigan Corporation, San Jose, CA.)

as it exits the tip of the metal capillary tube, thus producing a fine mist of highly charged droplets. At this point, two of the four fundamental parameters of ESI ion production have occurred. The first step involves production of the high electric field at the very narrow tip (100 μm) of the charged metal needle, followed by the transfer of this electric field to the electrolytic LC effluent that is in the process of being nebulized.

As the charged mist leaves the tip of the ESI needle, and makes its way into the heated, low-pressure region in the source, the positive charge applied by the metal needle creates an accumulation of the excess positive charge on the surface of the droplets that form the mist. The positive charge is drawn out, but cannot escape the surface of the liquid, and forms what is known as a Taylor cone. Since the simultaneous application of heat is continuously reducing the diameter of the droplets in the mist, the Taylor cone is stretched to a critical point, at which the charge escapes the liquid surface, and is emitted as an ion in a process known as a coulombic explosion. These two steps, i.e., the formation of the Taylor cone, followed by the coulombic explosion, complete the final two parameters of the four-step ESI ionization process.

One of the many advantages of the ESI process is its ability to generate multiple charged ions and tolerate conventional HPLC flow rates. Proteins and other large polymers (e.g., between 2000 and 70,000 daltons) can be easily analyzed on LC–MS systems having a mass limit of m/z 2000, due to this multiple charging phenomenon. For example, Interleukin-8 (rat) has a mass of 7845.3 daltons, but develops up to +8 charges, with the most abundant ion appearing at the +4 charge state (i.e., 7849.2 ÷ 4 charges = 1962.3 m/z), and thus can be analyzed on a LC–MS system having a mass limit of m/z 2,000. Powerful software can process in excess of +50 charge states, to yield the molecular ion information for larger proteins.

29.5.3 Atmospheric Pressure Chemical Ionization (APCI)

The atmospheric pressure chemical ionization interface is normally used for compounds of low polarity and some volatility. It is harsher than ESI and is a gas phase ionization technique. Therefore gas phase chemistries of the analyte and solvent vapor play an important part in the APCI process.

Figure 29-14 shows the schematic diagram of a APCI interface. The LC effluent-carrying fused-silica capillary tube protrudes about halfway inside a silicon–carbide vaporizer tube. The vaporizer tube is maintained at approximately 400–500°C, and serves to vaporize the LC effluent. High voltage is applied to a corona needle positioned near the exit of the vaporizer tube. The high voltage creates a corona discharge that forms reagent ions from the mobile phase and nitrogen nebulizing gas. These ions react with the sample molecules (M) and convert them to ions. A common cascade of reactions occurring in the presence of water, nitrogen gas, and the high voltage corona discharge is as follows:

$$e^- + N_2 \rightarrow N_2^{+\cdot} + 2e^- \qquad [7]$$

$$N_2^{+\cdot} + H_2O \rightarrow N_2 + H_2O^{+\cdot} \qquad [8]$$

$$H_2O^{+\cdot} + H_2O \rightarrow H_3O^+ + OH^\cdot \qquad [9]$$

$$M + H_3O^+ \rightarrow (M + H)^+ + H_2O \qquad [10]$$
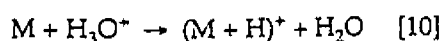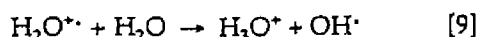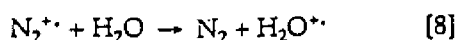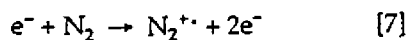
The APCI interface is a robust interface and can handle high flow rates of up to 2 ml/min. It is unaffected by minor changes in buffer strength or composition, and is typically used to analyze molecules less than 2000 daltons. It does not facilitate multiple charges, and hence cannot be used to analyze large biomolecules/polymers.

Improvements in LC–MS interfaces are continuously being made, although no universal interface for the various types of compounds is available yet. The
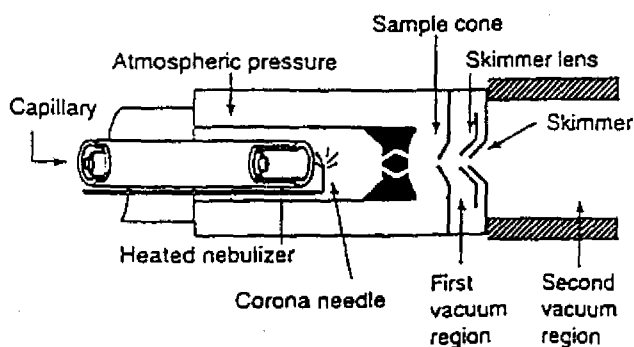
method is now becoming routine, although it is still difficult to compare results when using different types of interfaces. As technological advances are made, we can expect the use of LC–MS to grow, since the MS is a universal detector for both qualitative and quantitative information.

29.6 APPLICATIONS

The use of mass spectrometry in the field of food science is well established, but many areas of improvement are still in their infancy. While GC–MS has been used for years, only recently have low-priced reliable units been available as standard lab instrumentation. Routine use of GC–MS can be expected to grow as more public and private labs have access to the instruments. LC–MS, on the other hand, is still in the developmental stages, as researchers look for better ways to interface the two instruments and improve the ionization process. Nonetheless, a mass spectrum still can be obtained for most compounds eluting off an HPLC column, making the technique very useful.

There are many different applications of mass spectrometry in food science. One of the most thorough treatments on this subject is the book by John Gilbert listed in the resource materials section of this chapter. Readers should consult this book concerning specifics on a particular food component or a certain type of food. The coverage is excellent, although somewhat outdated since many developments have occurred since 1986.

In considering the application of GC–MS or LC–MS in food systems, note that if a compound can be separated by a GC or LC method, then chances are good that a mass spectrometer can be used. For years, the mass spectrometer was used only in a qualitative manner, to check the purity of eluting peaks or for compound identification. With smaller units, the use of MS as a universal detector has gained wide acceptance. The advantage of utilizing the MS as a detector is that only certain ions need to be monitored, which makes it a selective detector. This technique currently is used extensively for pesticide analysis.

The use of selected ion monitoring (SIM) is especially helpful in LC–MS, for which analysis is often limited by the lack of a suitable detector. Use of the SIM mode of detection is often the only way to detect some compounds eluting off the column. For example, fatty acids can be measured directly by HPLC, but unless the concentrations are high, an ultraviolet or refractive index detector will not pick them up. LC–MS will allow for the assay of trace amounts present in effluent. LC–MS has become especially helpful in the analysis of nonvolatile pesticides, amino acids, lipids, and sugars.

29-14 figure Schematic of an atmospheric pressure chemical ionization LC–MS interface. (Courtesy of Finnigan Corporation, San Jose, CA.)

29.7 SUMMARY

Mass spectrometry is fairly simple when examined closely. The basic requirements are to (1) somehow get the sample into a ionizing chamber where ions are produced, (2) separate the ions formed by magnets or quadrupoles, (3) detect the *m/z* and amount, and (4) output the data to some type of computer.

Since the qualitative and quantitative aspects of mass spectrometers are so powerful, they are routinely coupled to GCs and HPLCs. The interface for GCs is very versatile and easy to use. However, interfacing an MS to an HPLC still presents problems because there is no universal interface. We will continue to see developments in the HPLC interface, although the technology now exists to analyze many different types of compounds. Future developments will expand the use of mass spectrometry to just about any type of chromatographic separation method.

29.8 STUDY QUESTIONS

1. What are the basic components of a mass spectrometer?
2. What are the unique aspects of data that a mass spectrometer provides? How is this useful in the analysis of foods?
3. What is EI ionization? What is CI ionization?
4. What is a thermospray interface? What interface would you use for an HPLC run of a nonvolatile compound using a reversed-phase column, water, and methanol mobile phase?
5. What is the base peak on a mass spectrum? What is the molecular ion peak?
6. What are the major ions (fragments) expected in the EI mass spectrum of ethanol (CH_3—CH_2—OH)?
7. What are the major differences in how ionization occurs in the electrospray versus the APCI interface?
8. What are the major differences between the magnetic sector, quadrupole, and ion trap mass analyzer? What are the advantages of using each analyzer?

29.9 RESOURCE MATERIALS

Davis, R., and Frearson, M. 1987. *Mass Spectrometry*. John Wiley & Sons, New York. One of the best introductory texts on mass spectrometry. The authors start at a very basic level and slowly work through all aspects of MS, including ionization, fragmentation patterns, GC–MS, and LC–MS.

Gilbert, J. (Ed.) 1987. *Applications of Mass Spectrometry in Food Science*. Elsevier, New York. This review book gives thorough coverage to all the uses of MS in food science and of various food components.

Macrae, R. (Ed.) 1988. *HPLC in Food Analysis*, 2nd ed. Academic Press, New York. In addition to being a comprehensive reference on the use of HPLC in food analysis, this book contains a section (Chapter 13) on the application of LC–MS. Both instrumentation and applications are discussed.

McLafferty, F.W., and Turecek, F. 1993. *Interpretation of Mass Spectra*, 4th ed. University Science Books, Sausalito, CA (available from Aldrich Chemical Company, Milwaukee, WI). The fourth edition of an essential classic book on how molecules fragment in the ion source. Contains many examples of different types of molecules.

Niessen, W.M.A., and van der Greef, J. 1992. *Liquid Chromatography–Mass Spectrometry*, Marcel Dekker, New York. A thorough, though somewhat dated, review of all the LC–MS methods and interfaces.

Silverstein, R.M., Bassler, G.C., and Morrill, T.C. 1991. *Spectrometric Identification of Organic Compounds*, 5th ed. John Wiley & Sons, New York. An introductory text for students in organic chemistry. Chapter 2 presents mass spectrometry in an easily readable manner. Contains many examples of the mass spectra of organic compounds.

ACKNOWLEDGMENT

Magnetic Resonance

Thomas M. Eads

30.1 INTRODUCTION

Nuclear magnetic resonance (NMR) is a branch of spectroscopy that is useful for analysis because of the exquisite sensitivity of magnetic atomic nuclei (nuclear spins) to their environment. Electron spin resonance (ESR) is similar, but involves unpaired electrons (electron spins). NMR and ESR measure the magnetic properties of spins. This magnetic behavior is determined by molecular and ionic structure, motion, and interactions. These in turn are determined by chemical composition, distributions of mass among different phases (solid, viscous, liquid), molecular mobility (rotational and translational diffusion), and chemical and physical change in food materials. Thus NMR and ESR are the most versatile of all analytical tools.

ESR is used to detect free radicals that occur naturally in food, radicals produced in food by processing or irradiation, and naturally occurring paramagnetic ions. ESR also is used for measuring the physical state of molecules and molecular motion in foods, and can be used to measure oxygen concentration. Another name for ESR is EPR, electron paramagnetic resonance.

Food structure is of prime importance in all aspects of functionality. The spatially resolved versions of NMR (magnetic resonance imaging or MRI, and volume-localized spectroscopy or "in vivo NMR"), and ESR (electron spin resonance imaging) are powerful additions to the array of analytical food imaging techniques of photomicrography, and light, electron, and scanning probe microscopies.

Nuclei and electrons that are magnetic occur naturally in all matter, and thus can be observed easily in foods. They are observed via their resonance with electromagnetic radiation, just as electrons are observed by their resonance in ultraviolet (UV), visible (Vis), or infrared (IR) spectroscopy, except that the sample must first be immersed in a magnetic field. Because lower frequencies (radiofrequencies in NMR and microwave frequencies in ESR) penetrate food materials fairly well, the sample need not be optically transparent, in contrast to UV, Vis, and IR analysis (Chapters 26, 27). Because magnetic resonance "sees" the interior of the sample, it usually is not necessary to disrupt the sample beyond shaping it to fit in the magnet. This is a very powerful advantage.

The tradeoffs for magnetic resonance are that:(1) sometimes it does not have the sensitivity of other spectroscopic methods, (2) sometimes it does not have the selectivity of chromatographic methods, (3) the theory is a bit difficult, and (4) food applications require real strategy. These issues are yielding amazingly quickly to new theory, instrumentation, methods, and lively communication among users.

The standard methods of analysis of food materials include a few NMR methods such as moisture content (Chapter 8), oil content (Chapter 13), solid-to-liquid ratio (Chapter 14), or solid content. Even though a stunning array of magnetic resonance analyses has been reported for food materials, few have appeared in compendia of standard methods (see for example *ISO International Standards*, 1st ed., ISO 10565:1993(E), pp. 1–7). Progress in standardization is still somewhat slow.

The food analyst using magnetic resonance should ask the following questions:

1. What information do I want out of this sample?
2. What magnetic resonance techniques are appropriate?
3. What instrumental capabilities do I need to apply the techniques?
4. How do I interpret the results?
5. What other measurements do I need to complement the magnetic resonance data?

In this chapter you will learn how to answer these questions by learning vocabulary and principles, and the five important classes of analytical magnetic resonance measurements. An introductory bibliography is given at the end of the chapter.

30.2 PRINCIPLES OF MAGNETIC RESONANCE ANALYSIS

Analytical results depend very much on the chosen pulse sequence, instrument parameters, and sample condition. To optimize these, some basic theory is needed.

30.2.1 Immersion in a Magnetic Field (Fig. 30-1)

The sample is first inserted into a probe which is itself immersed in a strong magnetic field B_0 created by a permanent magnet (shown), electromagnet, or superconducting solenoid magnet.

30.2.2 Polarization (Fig. 30-2)

Nuclei (or electrons) spin like a top, and they also have charge. A spinning charge produces a magnetic moment μ, like a tiny bar magnet with a north and south pole. The magnetic moments of the spinning nuclei (or electrons) align in the strong magnetic field. Some align parallel to the static field B_0 and some align antiparallel. The slight excess of parallel creates the sample magnetization M, which is the vector sum over individual moments:

$$M = \Sigma \mu_i \qquad [1]$$

30-1 figure

30-2 figure

30-3 figure

$$\Delta E = E_2 - E_1 = h\gamma B_0/2\pi \qquad [3]$$

But we know from Equation [2] that $\gamma B_0 = \omega_L$, the Larmor frequency; thus

$$\Delta E = h\omega_L/2\pi = h\nu \qquad [4]$$

where h is Planck's constant. That is, a spin transition between "up" and "down" can be induced by absorption of a photon whose energy equals the difference in energy ΔE between the two spin states.

The energy is supplied by an oscillating magnetic field $B_1 \cos(\omega t)$ whose frequency has to be equal to the precession frequency ω_L, i.e., "on resonance," for absorption to occur. This is the origin of the term "magnetic resonance."

30.2.3 Precession of Magnetic Moments (Fig. 30-3)

The torque exerted by the static magnetic field on the spinning charges causes wobbling (precession) of the magnetic moment, much like the gravitational field causes a spinning top to wobble. Precession occurs at radiofrequencies (megahertz, MHz) for nuclei, and microwave frequencies (gigahertz, GHz) for electrons. The precession frequency is proportional to the field strength B_0:

$$\omega_L = \gamma B_0 \qquad [2]$$

where the proportionality constant γ, the gyromagnetic ratio, is specific for the observed nucleus. ω_L is known as the Larmor frequency.

30.2.4 Magnetic Resonance (Fig. 30-4)

Magnetic moments parallel to the magnetic field have slightly lower energy than those that are antiparallel. The difference is

30-4 figure

30.2.5 Excitation and Detection

In NMR the resonance field is produced by running a radiofrequency current through a wire coil (an inductor) that surrounds the sample. Both excitation and

detection occur through the same coil, which is part of the probe. In ESR the resonance field is supplied by microwave radiation transmitted through a wave guide into a resonator cavity that contains the sample.

30.2.6 Phase Coherence (Fig. 30-5)

Before the oscillating field is applied, magnetic moments are out of phase with each other and the component of M in the x–y plane, i.e., the transverse component, is zero. When the oscillating field is turned on, magnetic moments get in phase with each other, and the transverse component of M is now large.
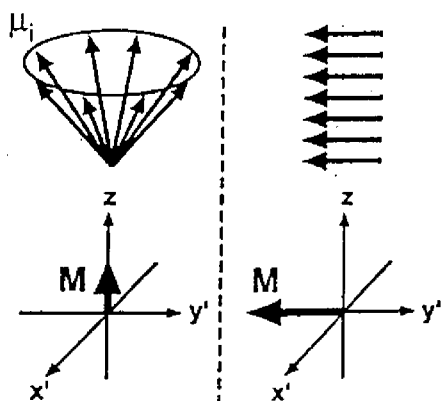
30-5
figure

30.2.7 Continuous Wave NMR and ESR (Fig. 30-6)

In continuous wave (CW) NMR and ESR, the excitation field B_1 (t) is weak. At resonance, nuclei (or electrons) align with B_1 and the sample absorbs measurably. The absorption spectrum is produced as the frequency of the oscillating field is swept. Alternately, the strength of the static field can be swept, while holding the oscillating field frequency constant. Field sweep is the common method for ESR.

30.2.8 Pulse NMR and ESR (Fig. 30-7)

In pulse NMR and pulse ESR the excitation field is strong, and is applied in a short pulse—a few microseconds. The magnetization M then precesses about B_1 as long as it is kept on. The time it is on is called the pulse width t_p. The angle of precession θ during this time is called the tip angle, which is calculated from the relation

$$\theta = \gamma B_1 t_p \qquad [5]$$

A rotation of θ = 90° puts the magnetization M right onto the transverse plane (x, y). This is called a 90°
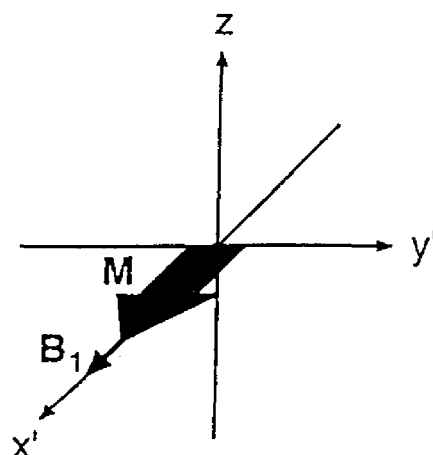
30-6
figure
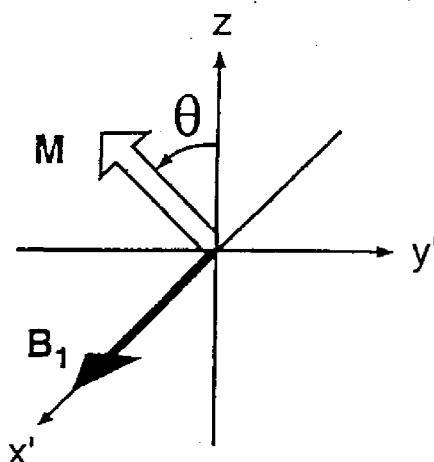
30-7
figure

pulse. A 180° pulse would invert M so that it now points along the minus z-axis. 90° and 180° pulses are used frequently in magnetic resonance.

30.2.9 The Induction Signal (Fig. 30-8)

After the 90° pulse (B_1 now turned off), M continues to precess about B_0 in the transverse plane. This produces an electromotive force (emf) or voltage in the measuring coil. For ESR, the perturbation of the electromagnetic energy in the resonator cavity surrounding the sample is measured. The principle of detection in pulse NMR is thus electromagnetic induction. The signal oscillates at the nuclear or electron precession frequency.

30.2.10 Relaxation and the Free Induction Decay

Following a pulse, M "relaxes," as shown by the spiral in Fig. 30-8. At any instant, the magnetization has a
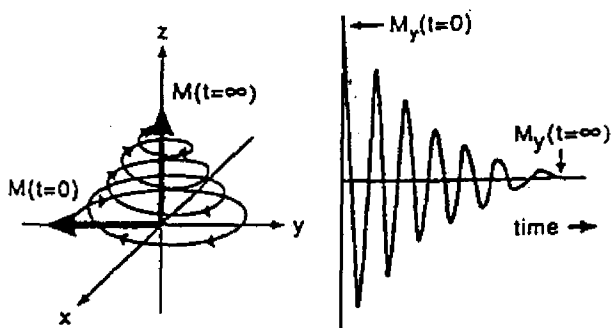
30-8 figure

30-9 figure

longitudinal component M_z, parallel to B_0, along the z-axis, and transverse component M_y in the plane perpendicular to B_0. The relaxing signal $M_y(t)$ is called the free induction decay or FID. The FID can be analyzed (see below), or it can be transformed from the time-domain to the frequency domain to give the NMR or ESR spectrum (see section 30.2.15).

30.2.11 Transverse or Spin–Spin Relaxation T_2 (Fig. 30-9)

The decay of the transverse component $M_y(t)$ is called transverse relaxation or spin–spin relaxation. The rate constant is T_2^*, which contains two contributions: the true nuclear (or electron) T_2, and the effect of inhomogeneity in the magnetic field, as given by the relationship

$$1/T_2^* = 1/T_2 + \pi \gamma \Delta B \qquad [6]$$

where $\gamma \Delta B$ is the spread in frequencies caused by field inhomogeneity ΔB. If this inhomogeneity is relatively small, then T_2 is related to the width of the resonance at half-height $\Delta v_{1/2}$ by the relation

$$T_2 = 1/\pi \Delta v_{1/2} \qquad [7]$$

If Δv_{inhomo} is relatively large, T_2 must be measured with a spin-echo pulse sequence, such as CPMG (for Carr Purcell Meiboom Gill) (Fig. 30-9). The spin–echo curve is analyzed as follows. Transverse relaxation is assumed to be a first-order process, i.e.,

$$d(M_{x,y})/dt = -M_{x,y}/T_2 \qquad [8]$$

where t is the decay time, which for this pulse sequence is $2\tau n$, where τ is the delay between the 90° and first 180° pulse, and n is an integer. First we separate the variables to get

$$\frac{dM_{x,y}}{M_{x,y}} = d \ln M_{x,y} = -\frac{1}{T_2} dt \qquad [9]$$
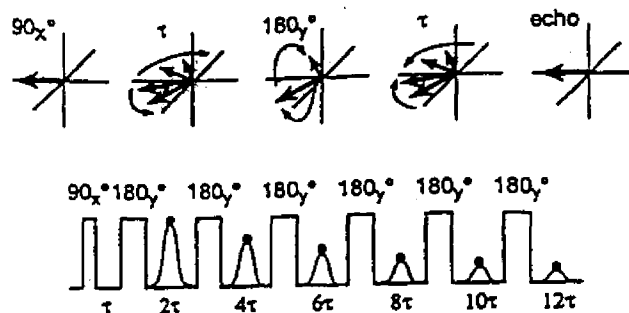
which can be integrated to give

$$\ln M = -\frac{t}{T_2} \qquad [10]$$

or

$$M(t) = \exp(-t/T_2) = \exp(2\tau n/T_2) \qquad [11]$$

Thus T_2 can be taken as minus the reciprocal of the slope of the straight line on a logarithmic plot, or obtained by fitting the echo decay data to the exponential function.

30.2.12 Solid, Viscous, and Liquid Transverse Relaxation (Fig. 30-10)

The decay of the signal from a solid domain is fast, since T_2 is short (about 5 μsec to 100 μsec), and so the line width (see Equation [3]) is very broad (about 60 kHz to 3 kHz). In this range, shorter T_2 means less mobility. Signals from viscous domains have T_2 from about 0.1 msec to 100 msec, and line width from 3 kHz to 3 Hz. In this range, shorter T_2 also means less mobility. The decay of the signal from a liquid domain is slow, since T_2 is long (about 0.1 sec to 1 sec), and so the resonance is narrow (about 30 Hz to 3 Hz). In this range, shorter T_2 can mean less mobility of liquid molecules. However, if liquid molecules are in contact with macromolecules such as proteins and polysaccharides, or with cell walls, membranes, or solid particles then the T_2 may become shorter. A shorter T_2 also may signal the presence of paramagnetic ions such as manganese, iron, or copper. Finally, a shorter T_2 may mean that chemical exchange is occurring, such as the exchange of protons between water and sugar hydroxyl groups.

30.2.13 Longitudinal or Spin–Lattice Relaxation T_1 (Figs. 30-11, 30-12)

After a pulse, the longitudinal component M_z also returns to equilibrium M_0. This is called spin-lattice relaxation or longitudinal relaxation, and the time constant is T_1. The longitudinal or z-component cannot

30-10 figure

Crystalline — Gaussian — 50 μs
Glassy
Viscous — exponential — 1 ms
Liquid — exponential — 500 ms

30-11 figure

M_0 180°   τ   90° $M_y(\tau)$ $-M_0$ $M_z(\tau)$

180°   90° $M_y(\tau)$ τ/s

30-12 figure

log T_1 $\omega_0\tau_r = 1$

10^{-4} 10^{-2} 10^0 10^2 10^4
$\omega_0\tau_r$

mobility	slow	intermed	fast
state	solid	viscous	liquid
temperature	low		high
interaction		more	less

30-13 figure

A

Intensity   0   Time / s

B

chemical shift δ = 8.0 ppm

area = $\int I(\delta)d\delta$

width at half-height = $\Delta\delta_{1/2}$ (ppm)

10   8   6   4   2   0
chemical shift / ppm
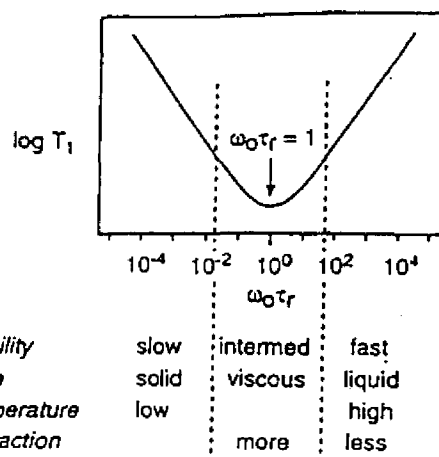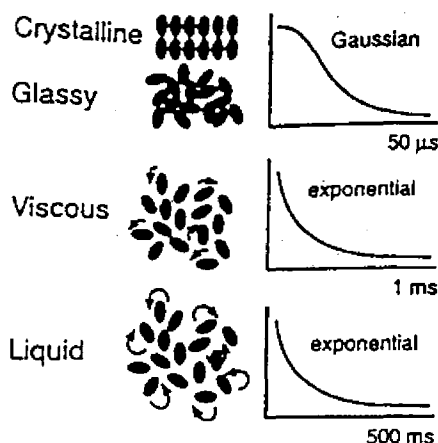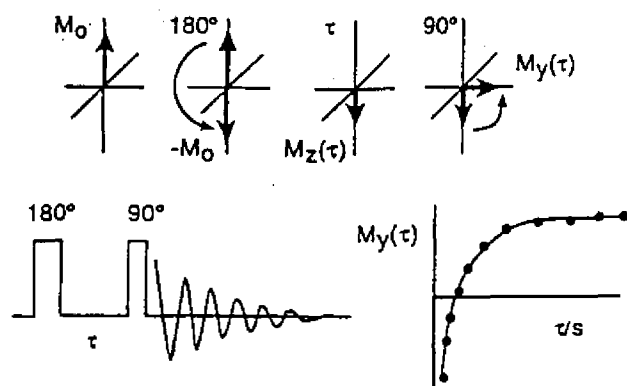
be measured directly because the receiver is in the xy plane. Instead, the inversion recovery pulse sequence is used (Fig. 30-11).

Longitudinal relaxation is assumed to be a first-order process, i.e.,

$$dM_z/d\tau = -[M_z(\tau) - M_0]/T_1 \qquad [12]$$

which can be integrated to give

$$M_z(\tau) = M_0[1 - 2\exp(-\tau/T_1)] \qquad [13]$$

Thus T_1 can be obtained by fitting the recovery data to the exponential expression, or by taking minus the slope of the plot of ln $[(M_0 - M_z(\tau))/M_0]$ versus τ.

T_1 is a measure of both molecular mobility and molecular interactions. The dependence on mobility shows a minimum (Fig. 30-13). However, on the liquid side of the minimum, a shorter T_1 also can mean more interaction between liquid molecules and macromolecules or solid surfaces if these are present.
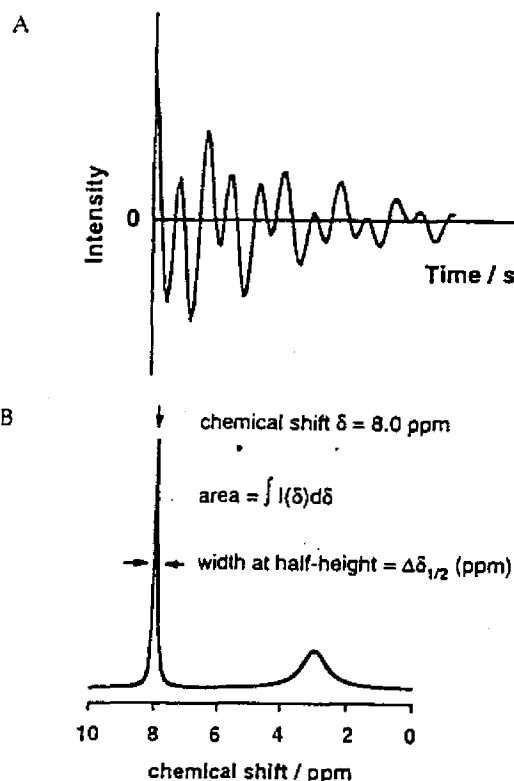
30.2.14 Observable Nuclei

For a nucleus to be observable by NMR, it must be a magnetic isotope, and the instrument must be able to detect it. A nucleus is magnetic if the nuclear spin quantum number I is nonzero.

Higher detection sensitivity depends on the nucleus (higher gyromagnetic ratio and the natural iso-

topic abundance) and on the instrument (higher magnetic field). The most sensitive food nuclei are hydrogen-1 (^{1}H, or "proton"), and phosphorus-31 (^{31}P). These have large gyromagnetic ratios and high natural isotopic abundances. The NMR parameters for isotopes most commonly found (or substituted) in food are listed in Table 30-1.

Nearly all molecules in food contain hydrogen and carbon atoms. Most bench-top NMR analyzers have weak magnets, and detect only ^{1}H. Most high resolution instruments have strong magnets and are equipped to detect other nuclei. Carbon-13 (^{13}C) is relatively insensitive due to a low isotopic abundance (1.3%) and small gyromagnetic ratio. But the ^{13}C spectrum is more spread out (over 200 ppm) than the ^{1}H spectrum (10 ppm), so resolution is better even if sensitivity is not. ^{13}C analysis is routine on high field magnets and is applied commonly to food liquids. However, NMR analysts are just learning how to measure ^{1}H, ^{31}P, and ^{13}C signals from physically heterogeneous food materials and very complex food liquids.

30.2.15 The High Resolution Spectrum (Fig. 30-13)

The time-domain signal (FID) in pulse NMR (Fig. 30-13a) or ESR can be Fourier-transformed to extract chemical information. If the magnetic field is very homogeneous, the resulting spectrum is called a high resolution spectrum (Fig. 30-13b). The basic information for each resonance is its position or chemical shift, its area, its width, and its multiplicity.

30.2.16. Chemical Shift and Spin–Spin Coupling

The high resolution spectrum may have many different resonances. Because nuclei in molecules are shielded from the magnetic field B_0 by electrons, they experience an effective field

$$B_{eff} = B_0 - \sigma B_0 = (1 - \sigma)B_0 \qquad [14]$$

where σ is dimensionless. The value of σ is of the order of 10^{-5} (10 ppm) for ^{1}H, and 2×10^{-4} (200 ppm) for ^{13}C. The new resonance frequency is now

$$v = (\gamma/2\pi)(1 - \sigma)B_0 \qquad [15]$$

which tells us that resonance frequency is proportional to the shielding factor $(1 - \sigma)$. Thus, nuclei that are chemically nonequivalent, i.e., belong to different chemical functional groups, also are magnetically nonequivalent, and give separate resonances in the spectrum. This is the basis for NMR chemical analysis.

In NMR, frequency is converted into chemical shift in parts per million (ppm) using the formula

$$[(v_{signal} - v_{reference})/v_{reference}] \times 10^6 = \delta(ppm) \qquad [16]$$

so that spectra can be compared from instruments having different magnetic field strengths B_0. The chemical shift range for protons is about 10 ppm, and for ^{13}C about 200 ppm.

Resonances may be split into multiplets by magnetic coupling with nearby nuclei (spin–spin coupling or J-coupling). Splitting also occurs in ESR due to cou-

30-1
table   Parameters for NMR-Active Nuclei in Food Materials

Isotope	Spin	Natural Abundance (%)	Relative Sensitivity[1]	NMR Frequency (MHz) at a Field of 2.3488 Tesla[2]
^{1}H	1/2	99.95	1.00	100.000
^{2}H	1	0.015	9.65×10^{-3}	15.351
^{13}C	1/2	1.108	1.59×10^{-2}	25.144
^{14}N	1	99.63	1.01×10^{-3}	7.224
^{15}N	1/2	0.37	1.04×10^{-3}	10.133
^{17}O	5/2	0.037	2.9×10^{-2}	13.557
^{19}F	1/2	100.00	0.83	94.077
^{23}Na	3/2	100.00	9.25×10^{-2}	26.451
^{25}Mg	5/2	10.13	2.67×10^{-3}	6.120
^{31}P	1/2	100.00	6.63×10^{-2}	40.481
^{35}Cl	3/2	75.53	4.70×10^{-3}	9.798
^{39}K	3/2	93.10	5.08×10^{-4}	4.667
^{43}Ca	7/2	0.145	6.60×10^{-3}	6.728
^{55}Mn	5/2	100.00	0.18	24.664
^{63}Cu	3/2	69.09	9.31×10^{-2}	26.505

[1] At constant field for equal numbers of nuclei. Multiply by the natural abundance to calculate the absolute sensitivity.
[2] Using the Larmor equation $\omega = 2\pi v = 2\pi/\beta$, these values for v can be used to calculate the gyromagnetic ratio.

pling of an unpaired electron with nearby nuclei, and this enables signals from different free radicals and paramagnetic ions to be distinguished from one another, and sometimes to be identified.

30.2.17 Effect of Field Strength (Fig. 30-14)

The separation between resonances increases with field strength. Greater separation means better selectivity. Then, more compounds can be quantified. Two resonances are resolved when the line width at half height $\Delta v_{1/2}$ is smaller than the chemical shift separation, $\delta_1 - \delta_2 = \Delta\delta$. Using Equation [6], we see that the condition for resolution is, thus

$$\Delta\delta > \Delta v_{1/2} = 1/\pi T_2^* = \frac{1}{\pi T_2} + \gamma \Delta B \qquad [17]$$

Sensitivity also increases with higher field. These analytical advantages of higher fields do not necessarily apply to ESR.

30.2.18 Summary for Magnetic Resonance Spectroscopy

Each peak in a spectrum has four characteristics used in analysis:

1. Chemical shift (for nuclei) or field value (for electrons), which contains general chemical information
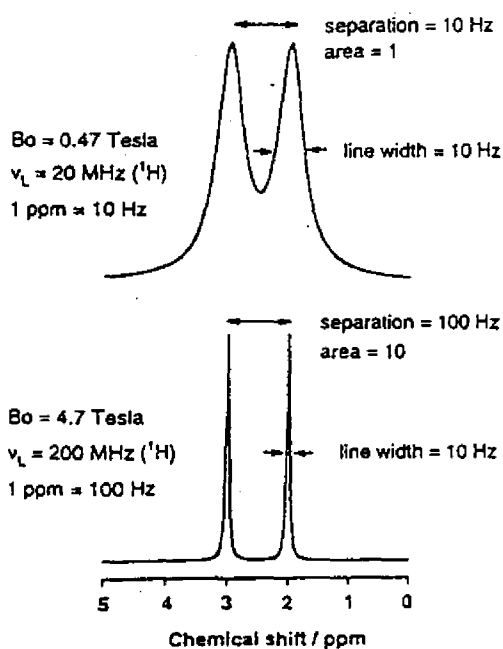
2. Multiplicity and splittings, which contain information about bonding patterns

3. Line width, which contains information about mobility, interactions, and field inhomogeneity

4. Area, which is proportional to the number of spins.

The combination is the basis for nondestructive chemical analysis.

30.2.19 Sample Requirements

The food sample can be virtually anything. This includes liquids (e.g., syrup, beverage, oil), semisolids (salad dressing, cheese, gel, meat, emulsion, margarine, etc.), or solids (milk powder, sugar granules, crackers, chocolate, etc.). Sample size is limited by the the magnet–coil combination. In most bench-top analyzers, the sample is between 1 and 50 cm^3; high resolution spectrometers, between 0.1 and 20 cm^3; ESR spectrometers, between 0.1 and 5 cm^3; imaging spectrometers, between 0.1 and 2.5 × 10^5 cm^3.

There are five main classes of magnetic resonance measurements used in food analysis, which are summarized in Table 30-2 and discussed in greater detail in the remaining sections of this chapter.

30.3 RELAXOMETRY (MOBILITY AND PHASES)

30.3.1 Description and Applications

The simplest class of magnetic resonance measurements, relaxometry, is also known as time-domain NMR, pulsed NMR, wide-line NMR, and low resolution NMR. In relaxometry the shape of a relaxation curve is the basis for analysis. Relaxation curves are of the four main types described below.

30.3.1.1 Free Induction Decay (FID)

The time constant T_2^* for the free induction decay of transverse magnetization M_{xy} recorded just after a pulse (see sections 30.2.8, 30.2.9, 30.2.10 and 30.2.12) is a general indicator of molecular mobility: slow molecular motion (as in solids) produces a short T_2, fast molecular motion produces a long T_2. If both solid and liquid are present, their signals superimpose. The two decays are easily distinguished because of the thousandfold difference in rates. This makes FID analysis useful in measurements of solid, viscous, and liquid mass in a sample. An important variation, the solid echo pulse sequence, preserves the proper shape of the solid decay, which is a Gaussian, not an exponential, thus avoiding an underestimate of solid T_2.

separation = 10 Hz
area = 1

Bo = 0.47 Tesla
v_L = 20 MHz (^{1}H)
1 ppm = 10 Hz

line width = 10 Hz

separation = 100 Hz
area = 10

Bo = 4.7 Tesla
v_L = 200 MHz (^{1}H)
1 ppm = 100 Hz

line width = 10 Hz

5   4   3   2   1   0

Chemical shift / ppm

30-14
figure

Table 30-2 Classes of Magnetic Resonance Measurements

Class	Field Strength	Field Homogeneity	Forms of Data
NMR relaxometry (time-domain, pulse, or low resolution NMR)	0.2–2.0 Tesla (8.5–85 MHz)[1]	Low (>15 ppm)	Free induction decay (T_2^*); decay of spin echoes (T_2); magnetization recovery curve (T_1)
Pulsed field gradient NMR (diffusion)	Same	Low (>15 ppm) to high (<0.01 ppm)	Attenuation curve (decay of spin echoes in presence of an applied magnetic field gradient)
High resolution NMR spectroscopy	0.5–21 Tesla (21–900 MHz)	High (<0.01 ppm)	As for relaxometry, plus: NMR spectra
Magnetic resonance imaging and volume-localized spectroscopy	0.1–14.1 Tesla (4–600 MHz)	Medium (<1 ppm) to high (<0.01 ppm)	One-, two-, or three-dimensional array of voxel intensities: images
Continuous wave ESR spectroscopy	0.3–0.6 Tesla (GHz)[2]	Low	ESR spectrum

[1]Proton (^{1}H) resonance frequency at the given field strength.
[2]Electron resonance frequency at the given field strength.

30.3.1.2 Spin–Echo Decay

The full liquid transverse decay is not seen in a FID because field inhomogeneity interferes (see section 30.2.11). This can be gotten around by using a repeating sequence of pulses such as in the CPMG method (see section 30.2.11). The resulting spin–echo decay curve, which is often multiexponential, gives the complete transverse relaxation, and the time constants are the true T_2s of any liquid components present. This makes spin–echo decay measurements useful for distinguishing oil from water in a sample, for separating several nonmixing water populations, and in measurements of molecular mobility in liquid and viscous phases.

30.3.1.3 Magnetization Recovery Curve

This is the familiar T_1 measurement. The return of longitudinal magnetization to equilibrium (along z) is measured in an inversion-recovery pulse sequence (see section 30.2.12, Equations [8–10]). The resulting magnetization recovery curve is often complex, but like complex transverse decay, this must not be ascribed automatically to separate "pools" of molecules having different mobilities. The mechanisms that give rise to such complex curves (dipolar coupling, chemical exchange) are beyond the scope of this chapter, but they happen to be most effective in food semisolids, suspensions, and liquids with appreciable concentrations of dissolved macromolecules (such as casein in milk). In the absence of these mechanisms, as in some food liquids, the time constant T_1 is shortest when the rates of molecular motions are close to the Larmor frequency $\omega_L = \gamma B_0$, which is fast (megahertz) (see Fig. 30-12). This

is why the T_1 shows a minimum as temperature is varied. T_1 measurements sometimes are correlated with liquid chemical composition using statistical comparisons, but this method should be used only with great caution on heterogeneous (semisolid) samples.

30.3.1.4 Attenuation Curve (Pulsed Field Gradient NMR)

When magnetic field gradients are applied to the sample before and after the 180° pulse in any spin–echo sequence, the echo decay now reflects diffusion rather than just T_2. That is, when a molecule moves by diffusion to a new position where the magnetic field is different, its frequency changes, and it gets out of phase, and the transverse signal decreases. The pulsed field gradient method is fully described in section 30.5.

The methods and applications of relaxometry in food analysis are summarized in Table 30-3. In an important variation of relaxometry, any of the above relaxation curves may be transformed into a continuous distribution of relaxation times instead of fitting to a sum of discrete exponentials. This data processing procedure is preferred for structurally heterogeneous materials and is found as a software option on modern benchtop NMR analyzers.

30.3.2 Instrumentation for Relaxometry (Fig. 30-15)

Relaxometry is usually performed on bench-top instruments equipped with permanent magnets or small electromagnets. Continuous wave (CW) spectrome-

30-3
table

Methods and Analytical Applications of NMR Relaxometry

Relaxation Curve	Pulse Sequence	Form of Data	Fitting Function	Applications
Free induction decay (FID)	Single pulse $(90°_x$ − acquire)	Complex decay of transverse magnetization (transverse magnetization versus time)	$M_{x,y}(t) =$ $\Sigma A_i \exp(-t/T_{2i})$ A_i is amplitude or mass fraction of ith decay component T_{2i} is rate constant of ith decay component	Moisture, oil, solid, or liquid content Solidity of solid phase, viscosity of viscous or liquid phase Solid/viscous/liquid ratio Solid fat content (SFC) Crystallization/dissolution Plasticization/glass formation Hydration/drying Freezing/thawing Unfrozen water in frozen systems; monolayer and multilayer water in low moisture systems
Solid echo decay	Solid echo $(90°_x - \tau_1 -$ $90°_y - \tau_2$ - echo)	Second half of echo decay	Gaussian-sinc decay for solid; Gaussian-exponential decay for solid + liquid	As for FID
Spin-echo decay	(CPMG) Carr Purcell Meiboom Gill $(90°_x -$ $[\tau - 180°_y -$ τ (echo) − $]_n$ Hahn spin-echo	Echo train, from which decay of echo amplitudes is extracted (echo magnitude versus time)	$A(t) = \Sigma A_i \exp(-t/T_{2i})$ A_i is amplitude of ith decay component T_{2i} is transverse relaxation time of ith decay component	Mobility in liquid or viscous phase Mobility distributions Oil/water ratio Unsaturation in oil Sugar concentration Ethanol concentration
Magnetization recovery curve	Inversion-recovery, $(180° - \tau - 90°_x -$ acquire) or progressive saturation	Magnetization recovery curve (z-magnetization versus delay between 180° and 90° pulses)	$M_z(\tau) = \Sigma M_{0,i}[1 -$ $2\exp(-\tau/T_{1i})]$ $M_{0,i}$ is amplitude of ith recovery component T_{1i} is longitudinal relaxation time of ith recovery component	High frequency motion in liquid, viscous, solid phases Interactions of liquid with solid Correlation with chemical composition, solids content, etc.
Echo attenuation curve	Pulsed field gradient with Hahn spin-echo or stimulated echo (Fig. 30-19)	Plot of decay of echo amplitude versus gradient strength	ln [$S(2t)/S(0)$] = $-\gamma^2 g^2 \delta^2 D (\Delta - \delta/3)$; where D is diffusion coefficient (see section 30.5 in the text)	Diffusion constant of mobile species Droplet size distribution in emulsions Pore size distribution in semisolids

ters and pulse-type spectrometers are marketed commercially. The pulsed NMR instruments are far more versatile; all the measurements listed in Table 30-2 can be done. Newer instruments are more automated, and can be integrated with robotics and laboratory information management systems (LIMS).

Magnets used for relaxometry need not be very homogeneous as long as chemical information is not required. To sort out which chemical species are contributing to the relaxation signal, it is necessary to do Fourier transform NMR (see section 30.4).

| computer |
| pulse programmer |
| transmitter |
| receiver/ADC |
| gradient driver |
| memory |

storage
display
printer

rf coils
gradients
magnet
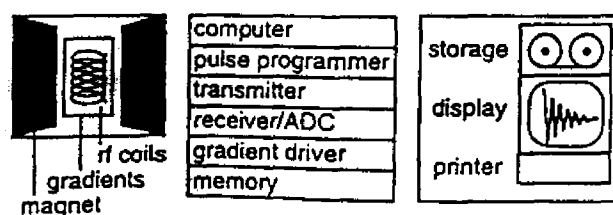
30-15
figure

Schematic diagram of a low field, low resolution pulse NMR instrument for relaxometry measurements.

Electronics for relaxometry includes frequency synthesizer, pulse programmer, transmitter including radiofrequency amplifier, probe including radiofrequency coil for excitation and detection, receiver including filters, and analog-to-digital converter.

The data system for relaxometry is a computer that controls electronics, data acquisition, and data processing. The data system also includes display (e.g., liquid crystal or video monitor), data transfer mechanism, and data storage devices (magnetic or optical disk, or magnetic tape).

30.3.3 Example: Solid Fat Content (Fig. 30-16)

A large difference in transverse relaxation rates in a FID distinguishes solid from liquid in measurements of solid fat content (SFC). The Fourier transform of the FID, although not usually calculated, would be a superposition of a very narrow resonance (liquid) upon a very broad resonance (solid). This spectrum is what is acquired directly in the CW NMR determination of SFC.

Figure 30-16 shows the principle of the "direct" SFC method, in which signal intensity after a 90°C pulse is sampled after the coil and receiver have calmed down (about 10 μsec) and at a later time (70 μsec). At 10 μsec, the signal contains solid plus liquid, and at 70 μsec, liquid only. Even at 10 μsec the solid signal already has decayed significantly while waiting for the probe to recover from the pulse, thus the method requires calibration with a set of samples of known solid content. SFC is calculated as

$$SFC = \%\text{ solid} = \frac{\text{protons in solid domain}}{\text{total number of protons}} \times 100$$

$$= 100\% \times \frac{S_A}{S_s + S_L} = \frac{fS_s}{fS_s - S_L} \qquad [18]$$

where f is the calibration factor. In the "indirect" SFC method, only the liquid intensity S_L is measured, and SFC is interpolated from a calibration curve obtained on the standards.
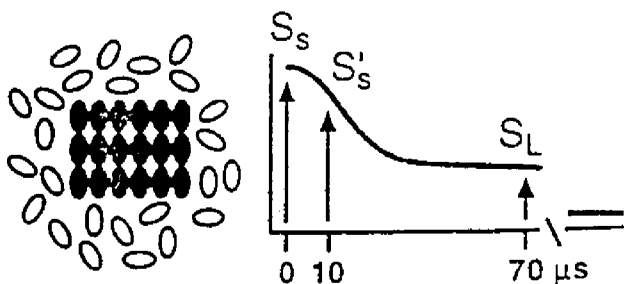
Principles of solid fat content analysis by NMR relaxometry. See the text for details.

30.4 HIGH RESOLUTION NMR (CHEMICAL ANALYSIS)

30.4.1 Description

When chemical information is desired, it is necessary to use high resolution CW NMR or pulse-Fourier transform NMR, and arrange for line widths to be smaller than the chemical shift differences between resonances (see section 30.2.17, Equation [16]). This means stronger and more homogeneous magnets (see sections 30.2.14, 30.2.15, 30.2.16). In rapid scan correlation NMR (a variant of CW NMR), signal averaging is employed to increase the signal-to-noise ratio. In pulse NMR, FIDs are summed for the same purpose. Rapid scan correlation NMR has certain advantages over pulse FT NMR for routine analysis of liquid phases in food materials by ^1H and ^{13}C NMR.

NMR is well known for its use in total chemical structure determination of isolated compounds of molecular masses up to about 20,000 kilodaltons, and numerous applications to food components ranging from sugars to lipids to flavor and color compounds and even proteins have been reported. This application is not considered further here, but it is useful to remember that the structural methods also can be applied to sort out the hundreds of resonances in NMR spectra of very complex food liquids.

Analytical high resolution NMR requires that the time between scans (or pulses) is longer than five times the longest analyte T_1. Otherwise, the magnetization M_z does not recover completely, and the final signal is smaller than its true or equilibrium value. This effect is called saturation. Intensity and chemical shift standards also should be incorporated into samples, by direct addition or by inserting a capillary containing the standard. Identification of resonances is achieved by comparison (often computer-automated) with standard spectra of purified compounds, many of which are found in published compilations, just as for infrared spectroscopy and mass spectrometry. Resonances also may be identified from the empirical rules for chemical shifts and J-coupling, which are very well known, and appear in the basic texts on NMR spectroscopy.

The five main types of high resolution spectra in food analysis are described below and in Table 30-4.

30.4.1.1 Single Pulse Spectra of Liquid Phases

This signal is obtained by taking the Fourier transform of the free induction signal following a single pulse whose tip angle is 90° or less. This measurement selects liquid phase components including water, oil, and substances dissolved in those phases. However, structural, and hence magnetic, inhomogeneity in the sample can

30-4

Methods and Analytical Applications of High Resolution NMR

Type	Pulse Sequence	Line Narrowing	Form of Data	Applications
Single pulse/liquids	Single pulse	Magic angle spinning (MAS) helpful if sample is heterogeneous	One-dimensional spectrum (intensity versus chemical shift)	Analysis of aqueous or oil phase composition in liquid or semisolid foods Plus all relaxation times, molecular properties, and processes under relaxometry, but with chemical shift resolution
SNIF NMR (site-specific natural isotope fractionation	Multiple nuclei observed (e.g., ^1H, ^{13}C, ^2H) generally in single pulse experiments on compounds isolated from foods and beverages	Usually not needed	Multiple spectra; ratios of peaks are compared to determine isotopic ratio at each atomic position in a molecule	Detection of adulteration Authentication Tracking biological, biochemical, and geographic origin Forensics
Proton-decoupled/ liquids	Single pulse in observe channel (e.g., ^{13}C); CW or pulse train in decoupling channel (e.g., ^1H)	Low power decoupling	One-dimensional spectrum (intensity versus chemical shift)	As above, however, the spectra are simplified, better resolved, and have higher SNR
Two-dimensional/ liquids	COSY, HETCOR, NOESY etc.	Low power decoupling; greater resolution by spreading data in more dimensions MAS if sample is heterogeneous	Topographic (contour) plot; axes in HETCOR, for example, are ^{13}C chemical shift and ^1H chemical shift	Analysis of complex liquids: fermented or distilled, milk, natural oils, processed oils, fruit juices, vegetable juices, extracts, etc.; also, liquid phases in food semisolids
One- or two- dimensional/solids	Cross polarization of abundant (e.g., ^1H) to rare (e.g., ^{13}C spins), then single pulse; various sequences for chemical shift exchange, and motion	High power proton decoupling to remove dipolar coupling; magic angle spinning to remove chemical shift anisotropy	One-dimensional spectrum (intensity versus chemical shift); or two-dimensional, with various axes	Analysis of composition of solid phases in solid and semisolid foods: protein, polysaccharide, crystalline fat, sugar Polymorphism in crystalline fats, sugars, confections
Total NMR spectrum	Solid echo	Short dead time, fast ADC (MHz), long acquisition; MAS to remove susceptibility broadening	One-dimensional spectrum; vertical expansion to show solid resonance; horizontal expansion to show liquid resonances	Simultaneous solid/viscous/liquid ratio and composition of liquid phase Phase transitions Molecular mobility distributions

broaden liquid resonances. The technique of low-speed magic angle spinning (MAS) recovers the resolution. The compositions of liquid phases in fruit, meat, emulsions, and even baked goods have been determined by this very powerful and rapid method of nondestructive analysis. An example is given in section 30.4.3.1.

30.4.1.2 Proton-Decoupled Spectra of Liquid Phases

The resonance of an observed nucleus like ^{13}C may be split due to spin-spin coupling with protons connected to it by chemical bonds. The resulting multiplets can be collapsed by continuously exciting the protons (at their

resonance frequency) during detection of the carbon signal. This is called low-power decoupling, and it results in simpler spectra, better resolution, and better signal-to-noise ratio, making chemical analysis much easier.

30.4.1.3 Two-Dimensional NMR Spectra of Liquid Phases

Liquid spectra often are crowded due to chemical complexity. By spreading this information over two or more dimensions, crowding is relieved. Many kinds of 2-D (and n-D) NMR pulse sequences accomplish this. Overlapping peaks are resolved, selectivity is vastly improved, and many more compounds can be analyzed. Nondestructive chemical analysis of food liquid phases by multidimensional NMR will become routine and perhaps an automated measurement in the future. These methods already are becoming important in food regulatory analysis such as authentication and detection of adulteration in fruit juices, oils, wines, etc. The method of site-specific natural isotope fractionation, or SNIF–NMR, accomplishes these goals by measuring deuterium and proton spectra, and calculating isotopic ratio at every molecular site. The pattern of ratios depends entirely on sample history.

30.4.1.4 High Resolution Spectra of Solid Phases

Broadening due to strong magnetic interactions in solid phases can be relieved by three line-narrowing techniques: high power decoupling, magic angle spinning, and multiple pulses. In addition, sensitivity can be enhanced by cross polarization. A description of the strong interactions and the line-narrowing techniques is beyond the scope of this chapter. However, these methods have become routine, and it is possible to obtain high resolution NMR spectra selectively from solid phases in intact solid and semisolid food materials, and to approximate the chemical composition of solid phases. An example is given in section 30.4.3.2.

30.4.1.5 Total NMR Spectrum

The total NMR spectrum, i.e., solid plus viscous plus liquid, can be obtained if the free induction decay (or solid echo decay) is acquired under special conditions: short pulses, detection within 4 µsec of excitation to capture the full solid decay, sampling rate faster than 1 MHz to properly digitize the solid decay shape, long detection time to capture full liquid decay, and magic angle spinning to eliminate inhomogeneity broadening of liquid resonances. The resulting spectrum has two major features. First is a very broad resonance, devoid of chemical information, but representing solid (and viscous, if present) which differ in their T_2s, and

hence in their line widths, by a factor of ten to one hundred. The second feature is a set of narrow resonances, a liquid spectrum, superimposed on the broad resonance, just a few percent as wide as the solid. The high resolution feature gives the composition of liquid phases. The ratio of the integral of the broad feature to the integral over all the narrow resonances gives the (solid + viscous)-to-liquid ratio.

30.4.2 Instrumentation for High Resolution NMR

High resolution NMR is done on instruments equipped with stable electromagnets or superconducting solenoidal magnets. Shim coils make the magnetic field extremely uniform so that at least one resolution criterion is met (section 30.2.17, Equation [16]). Continuous wave spectrometers and pulse-Fourier transform type spectrometers are marketed commercially. The pulsed NMR instruments are far more versatile, permitting spectral editing, multidimensional NMR, as well as relaxation analysis. Newer instruments are more automated, and can be integrated with robotics and laboratory information management systems (LIMS).

Electronics for high resolution NMR include multiple frequency synthesizers (for excitation of various magnetic isotopes), pulse programmer, transmitter with several channels, broadband radiofrequency amplifiers, field-frequency lock to keep the spectrometer on resonance, multichannel probes with electronics for tuning to multiple frequencies and radiofrequency coils for excitation and detection, broadband phase-referenced receiver including filters, and analog-to-digital converter. The probe quality factor (Q) usually is much higher for a high resolution probe than for a relaxometry probe.

The data system for high resolution NMR is a computer that controls electronics, data acquisition, and data processing. The data system includes data display, software for creating and modifying pulse sequences, a means for coordinating NMR with control of sample conditions and with robots, and transfer and storage devices. Off-line data processing using commercial software is common, since spectrometer computers are usually busy.

The rapid scan correlation instrument replaces CW NMR, the hardware having been modified for better resolution, higher signal-to-noise ratio, and greater speed of analysis.

30.4.3 Examples of High Resolution NMR Analysis

30.4.3.1 Analysis of Strawberry (Fig. 30-17)

Liquid domains in strawberry fruit include the aqueous intracellular and interstitial spaces, and oily
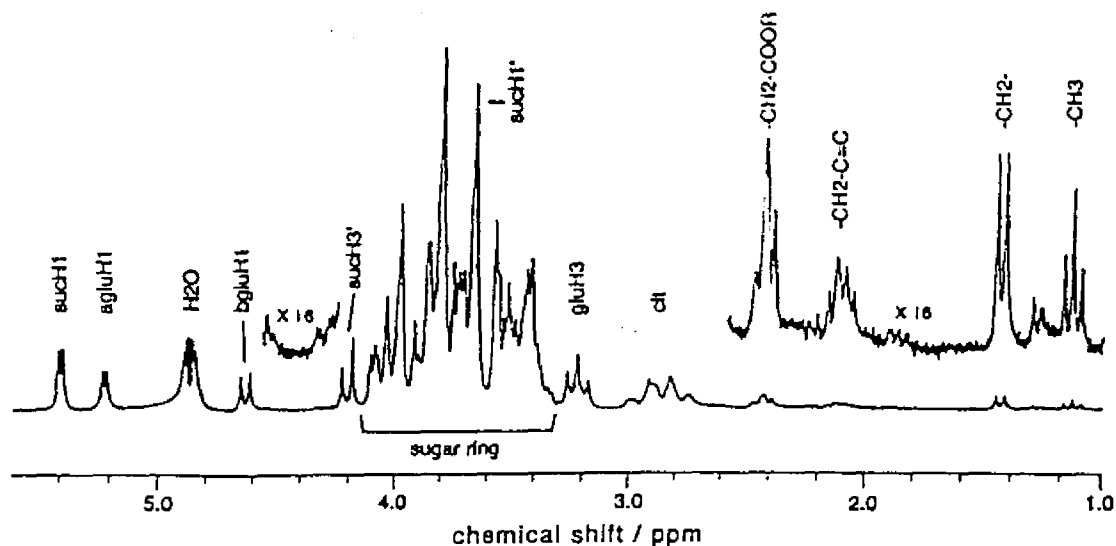
30-17 figure High resolution magic angle spinning proton 200 MHz NMR spectrum of liquid phase components of intact strawberry tissue, using DANTE pulse sequence for water peak suppression, and magic angle spinning (MAS) to recover resolution. From this spectrum, the composition of soluble components was estimated: sucrose, 1.5 g/100 g of tissue; d-glucose, 0.99; d-fructose, 3.28; l-ascorbate (Vitamin C), 0.027; l-malate, 0.011; citrate, 0.47. Amino acids and fatty acyl lipid resonances were identified but could not be estimated with confidence.

domains include lipid droplets and mobile regions of bilayer membranes. Molecules in liquid phases are highly mobile, giving a nice high resolution NMR spectrum, as shown in Fig. 30-17 for intact strawberry tissue. To get this spectrum, a small cylinder of tissue was cut from a fresh strawberry and placed into a rotor, which was then oriented at 54°44' (the "magic angle") relative to the magnetic field, then spun about its axis at several hundred Hertz. Resonances due to protons in different chemical functional groups of sugars, organic acids, and fatty acyl lipids were identified by comparison to spectra of pure compounds. The composition of the liquid phases of the sample was calculated from integrals. The NMR analysis was done without sample preparation beyond cutting a plug. There was no grinding, separations, or chromatographic procedures, or use of hazardous chemicals.

This high resolution spectrum is the "signature" of this particular piece of fruit, a direct result of species, variety, growing conditions, and storage history. This spectrum could be placed with others like it in a library of strawberry spectra. When a new spectrum is obtained, it can be compared with spectra in the library to identify its origin and history. Such methods are under intense development for their value in food science, nutrition, authentication, and processing.

30.4.3.2 Crystalline Sugars in Licorice (Fig. 30-18)

Before the spectrum in Fig. 30-18 was obtained, there were only hypotheses about which components in licorice, a semisolid confection, were in a solidlike

state. Licorice is made from wheat flour, oligosaccharides and sugars, water, emulsifiers, colorings, and flavorings. As it ages, it becomes tougher (even without moisture loss), and has less flavor impact. High resolution carbon-13 solids NMR revealed part of the reason: a substantial fraction of the sugars has crystallized. Assignments of sugars in crystalline state were made by comparison with spectra of pure crystalline compounds.

30.5 PULSED FIELD GRADIENT NMR (DIFFUSION)

30.5.1 Description

This method falls into the class of relaxometry (Table 30-3), but also is done on high resolution and imaging spectrometers. It requires special treatment. Translational motion of molecules, i.e., diffusion, is important in many food functionalities including processability, nutritional quality, sensory quality, stability, or food safety. For example, chemical reactions important in processing and shelf life often are diffusion limited. Processes such as crystallization, drying, and hydration are obviously limited by diffusion. The restricted diffusion in emulsion droplets, or in pores in low moisture or frozen food materials, has a signature that tells us something about the microscopic structure. Even bioavailability of nutrients in a bolus of material moving through the upper intestine depends on diffusion. Thus measurement of diffusion is becoming a more common analytical measurement.

chemical shift / ppm
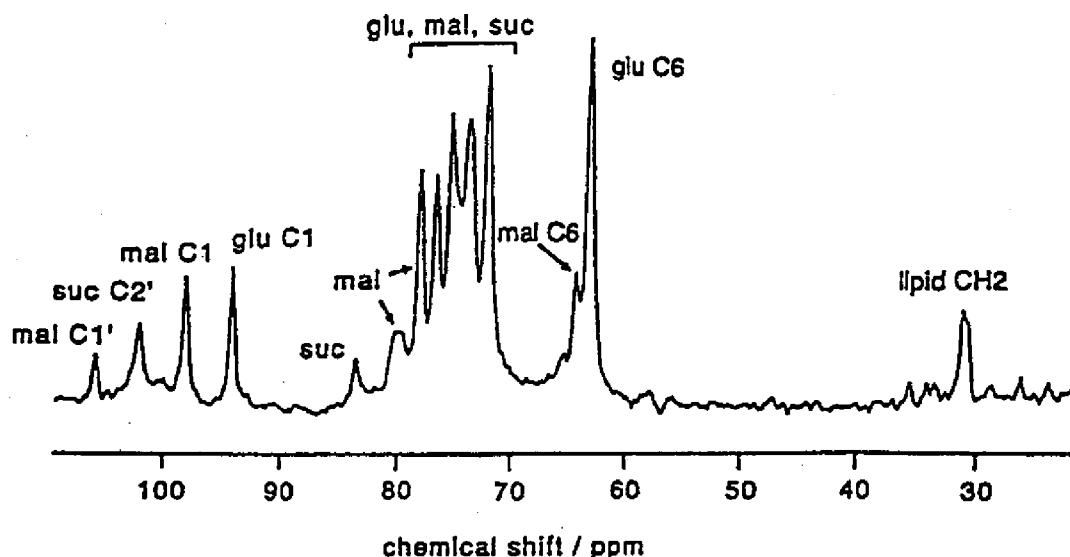
30-18 **figure** Carbon-13 50 MHz NMR spectrum of intact licorice, taken with sensitivity enhancement by cross-polarization (CP), and line-narrowing by magic angle spinning (MAS) at 1.5 kHz, and high power proton decoupling (HD). Resonances of ^{13}C atoms in sugars present in a crystalline state are identified by their chemical shifts. (Sample courtesy of Hershey Foods Corp.)

The principle of NMR diffusion measurement is that when a molecule moves to a place where the magnetic field is different, its resonance frequency will change. The field difference may be present already due to magnet inhomogeneity, or due to structures in the food. Thus a strong gradient is assured by application of a stronger magnetic field gradient. The change in frequency results in more rapid loss of transverse magnetization, which is easily measured in spin-echo experiments like those used to measure T_2. A typical method is called pulsed field gradient spin echo (PFGSE). Translational motions slower by four orders of magnitude than that of water (in water) can be measured with this technique, making it useful for viscous and semisolid foods, and for variable temperature studies. The PFGSE sequence employing the Hahn spin echo and an attenuation curve are shown in Fig. 30-19. An important variant, the stimulated echo pulse
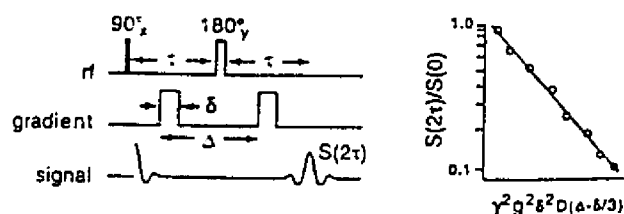
30-19 **figure** The principle of pulsed field gradient NMR (FFGNMR) measurement of diffusion coefficient in liquids. The pulse sequence is on the left, and a simulated attenuation curve on the right. The diffusion coefficient is obtained from the slope of the line.

sequence, is preferred for measurement of diffusion in viscous phases, and for determination of droplet size distribution and pore size distribution.

The experiment is repeated with different gradient strengths g. The data may be analyzed graphically according to the following expression

$$\ln[S(2\tau)/S(0)] = -\gamma^2 g^2 \delta^2 D(\Delta - \delta/3) \qquad [19]$$

where:

γ = gyromagnetic ratio
g = strength of the field gradient (Gauss/cm)
D = translational diffusion constant (cm^2/sec)
δ = duration (seconds) of the gradient pulse
Δ = diffusion time

This expression predicts that a plot of the natural logarithm of the attenuation versus g^2 will be a straight line with the slope proportional to the diffusion constant D. Alternatively, the data may be fit by computer to the above expression. Curvature may mean restricted diffusion, as within droplets in an emulsion (see the example in section 30.5.3). Use of PFGSE NMR requires calibration of the field gradient strength.

Some typical diffusion constants are: water in water at 20°C, $D = 2.3 \times 10^{-9}$ m^2/sec; water in cheese, 0.39×10^{-9}; triacylglycerol molecule in liquid milk fat, $D = \sim 0.01 \times 10^{-9}$. The diffusion constant is slower when temperature is lower, when viscosity is higher, or when the liquid phase is highly dispersed, as for example the moisture in bread.

30.5.2 Instrumentation for Pulsed Field Gradient NMR

All that is required for PFGSE NMR in addition to the basic instrumentation described for a benchtop relaxometer is a gradient coil that, like the rf coil, surrounds the sample, an amplifier to deliver the direct current to the gradient coil, and computer control of the start and end of gradient pulses. Commercial software for automating setup, calibration, parameter input, and data analysis has appeared recently. When high strength gradients are used, they may be actively shielded to reduce interference with the NMR measurement.

30.5.3 Example: Fat Droplet Size Distribution in Swiss Cheese

Droplet size distribution often is measured because it affects rheology, appearance, texture, flavor release, digestibility, and other properties of food emulsions. Coulter counting requires liquefaction and dilution, and conventional microscopy requires partial sample destruction. The NMR method, based on the PFGSE method described previously, makes the measurement on the intact sample. It is spreading rapidly as a method of choice. Some of the data from the first application of the method to food (Callaghan et al., 1983) are shown in Fig. 30-20.

Here we see that the low resolution NMR spectrum permits separate observation of fat and water signals in Swiss cheese. Attenuation curves [ln[S(2τ)/S(0)] versus g^2] were obtained once appropriate values of the gradient pulse strength g, duration δ, and τ had been found. The data for the water peak (not shown)

formed a straight line and were fit to the above expression (Equation [16]), and the result was $D(H_2O) = 3.9 \times 10^{-10}$ m^2 sec^{-1}. This is about one-sixth that of water in water at the same temperature (30°C), and suggests that water diffuses through a tortuous network on casein particle surfaces. The data for the fat signal formed a curve, indicating restricted diffusion, and were thus fit to the following expression

$$\ln[S(2\tau)/S(0)] = -\alpha^2 R_0^2(1 + \sigma^2\alpha^2)^{-1} - (1/2)\ln(1 + \sigma^2\alpha^2) \quad [20]$$

where:

R_0 = mean fat droplet radius
σ^2 = variance for a Gaussian distribution of droplet sizes ($\sigma/\sqrt{2}$ would be the standard deviation SD)
$\alpha^2 = \gamma^2 g^2 \delta^2/5$

The result for Swiss cheese was $R_0 = 2.65$ μm and $\sigma = 1.60$ μm. Clearly there is a wide spread in fat droplet sizes.

The above method used Fourier transform NMR for detection of the liquid signals, which are separated by chemical shift. Even if a low field, low resolution magnet were used, it would be easy to observe the fat signal separately. As it turns out for Swiss cheese, water proton T_2 is much shorter (12 msec) than the liquid fat methylene proton T_2 (40 msec). Thus by setting the value of t to be several times longer than the water proton T_2, only the fat signal would remain in the echo.

30.6 MAGNETIC RESONANCE IMAGING (STRUCTURE)

30.6.1 Description

There are two major kinds of spatially resolved NMR. The first, magnetic resonance imaging, or MRI, obtains an anatomical picture with resolution ranging from macroscopic to microscopic. The second, volume-localized spectroscopy ("in vivo" NMR), obtains a spectrum from a specific volume within a sample, the size of the sampled volume ranging from macroscopic to microscopic.

30.6.1.1 Magnetic Resonance Imaging (Fig. 30-21)

The following is a simplified description. The first step of imaging is to get every position in the sample to have a different resonance frequency, which is achieved by applying magnetic field gradients. This is called frequency encoding. The second step is to detect the NMR signal; this is achieved by doing NMR experiments such as single pulse or spin echo, and synchronized

Pulsed field gradient NMR measurement of fat droplet size distribution in Swiss cheese. Low resolution proton NMR spectrum shown on left; attenuation curve for fat signal on right. (Adapted with permission from Academic Press, Inc. From Callaghan, P.T., Jolley, K.W., and Humphrey, R.S. 1983. Diffusion of fat and water in cheese as studied by pulsed field gradient nuclear magnetic resonance. *Journal of Colloid Interface Science* 93:521–529.)

gradient

y

OBJECT

0   x

NMR SPECTRUM

0   frequency

90°x

time

radiofrequency
pulse

frequency-
encoding
gradient along x

NMR signal

30-21 figure

Principle of frequency encoding in magnetic resonance imaging. The NMR spectrum shown is actually a one-dimensional image or projection of spin density onto the gradient direction. A "slice" of the sample can be selected by using a specially shaped 90° pulse, and by applying additional gradients. Such two-dimensional images can be obtained very quickly for nearly any kind of food material.

with the gradients. The third step is to convert the NMR frequency back to position. This is called reconstruction. The result is a map, more or less faithful to the original structure.

Usually, the image is a two-dimensional slice of finite thickness, the plane being oriented in any desired direction. One-dimensional images are projections of the structure along one direction. Three-dimensional images also are useful. These have to be viewed as series of slices or as solid surfaces.

When resolution in an imaged plane is 100 μm or less, it is called NMR microscopy, or microimaging. The best routine microscopic resolution is a volume element (voxel) whose dimensions are $10 \times 10 \times 50$ μm, near the size of plant and animal cells. NMR microscopy is not as economical or resolved as other imaging methods such as light microscopy; it is more widely

used in food research than analysis. This situation is changing with the introduction of relatively low-cost bench-top imaging spectrometers. Very good imaging times and good resolution (0.5 mm) in food materials already have been demonstrated with prototypes. This is an exciting development with important applications for quality measurements.

The great advantages of MRI over other kinds of imaging are that it is nondestructive, it can "see inside" the sample, and the basis of contrast can be chosen by the analyst. The different contrast mechanisms, and the analytical quantities measured are presented in Table 30-5.

30.6.1.2 Volume-Localized Spectroscopy (In Vivo NMR)

When the analyst wants to know the chemical composition in a particular volume of a food sample, volume-localized spectroscopy is the method of choice. This method uses field gradients and special pulse sequences to force the NMR signal to originate from a selected volume. The resulting high resolution spectrum gives chemical composition in that volume alone. Usually ^{1}H spectra are obtained. However, advances in pulse sequences and detector sensitivity have made ^{13}C and other nuclei accessible by this method.

30.6.2 Instrumentation for Magnetic Resonance Imaging

Magnetic resonance imaging and volume-localized spectroscopy both require that the spectrometer be equipped with magnetic field gradient coils (x, y, and z), gradient drivers, and computer control over gradient strength, duration, and timing. The radiofrequency coils for volume localized spectroscopy usually do not completely surround the sample, but may be flat or curved, and placed on the surface of the sample while it is still in the static magnetic field. These are called surface coils. Advanced software makes it easy to set up and perform routine MRI, and to reconstruct and display images. Quantitative analysis is more challenging. MRI also has been sued to follow flow, temperature, phase changes, and structure changes in food during real processes.

30.6.3 Example: Oil, Water, and Sugar Images of a Grape (Fig. 30-22)

In Pope's classic MRI study of a grape, he produced images of lipid only (the seeds show up), sugar only (regions in the pulp with higher sugar content are brighter), and water only (the skin and seeds have less water than pulp). This was accomplished by using chemical shift imaging. Many different kinds of foods have been imaged this way.

30-5 table

Methods and Analytical Applications of Magnetic Resonance Imaging

Basis for Contrast	Analytical Quantity Imaged	Pulse Sequence (examples)	Form of Data	Applications
Spin density	Pure anatomy	2D-FT (spin-warp)	1D: graph of intensity versus position 2D: picture, where intensity is given a grayscale or color value 3D: "solid" array, usually projected onto two dimensions at a time Difference maps from a time series show change, e.g., diffusivity of moisture or salt	Anatomy, macrostructure, microstructure of intact foods Processes: hydration/dehydration; brining of muscle foods and cheeses; phase changes, foam collapse, hardening/ softening
Chemical shift or frequency	Chemical composition	CHESS	Separate images given for different selected resonances or compounds in liquid state	Localization of sweet regions in fruit Localization of lipid in processed meats
Molecular translational diffusion	Diffusion constant	STEAM	As for spin density, except that "brightness" corresponds to rate of diffusion	Find regions of high water or lipid mobility in any kind of food
Relaxation time weighting (T_1, T_2, T_{1r})	Anatomy with contrast enhanced by relaxation	Variations on spin-warp that select for species with relaxation times longer than some minimum	As for spin density	Highlight regions of high or low water mobility, or much or little water–macromolecule interaction
Relaxation time mapping (T_1, T_2, T_{1r})	Actual value of local relaxation time constant	T_1: inversion or saturation T_2: Hahn, Carr Purcell, stimulated echo for liquids T_2: Constant time or single point imaging (SPI)	Series of images corresponding to variable delay in pulse sequence; relaxation curve analyzed at each position; plot separate amplitude and time constant as pixel intensities; do this for each resolved relaxation component	Map of solidity in gels, bread, semicrystalline fat, confections, frozen foods etc.

30.7 ESR ANALYSIS (FOOD SAFETY)

30.7.1 Description

30.7.1.1 Rationale for Use of ESR

ESR detects organic free radicals, molecules in triplet states, and paramagnetic metal ions. Use of ESR in food analysis is important because:

1. High levels of free radicals and extensive oxida-

tion are nutritive and medical liabilities, and foods must be examined for their safety.

2. High levels of free radicals also are signs of improper use of ionizing radiation (such as gamma irradiation), abusive processing, or chemical or biological deterioration. Their presence indicates the need to change how the food is handled.

3. Not all countries permit sale of irradiated food-

water

oil

sugar

8 7 6 5 4 3 2 1 0
chemical shift / ppm

water image

sugar image   oil image
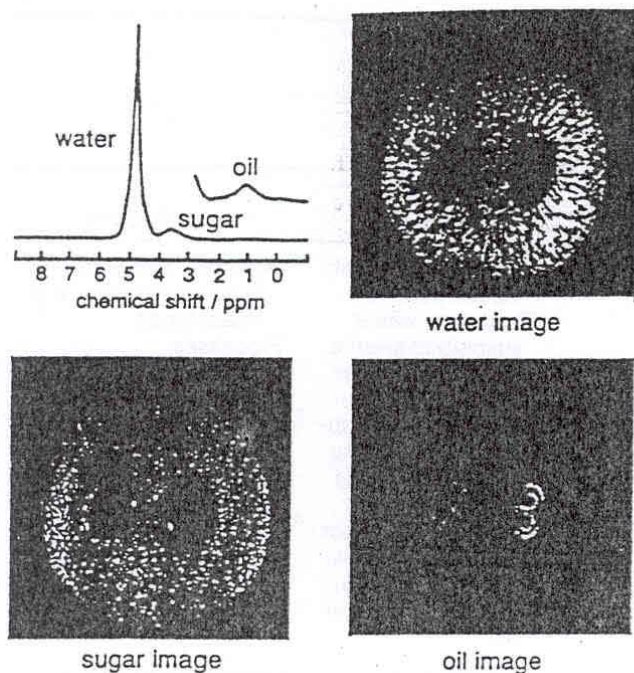
30-22
figure

Chemical shift images of water, oil, and sugar in an intact grape. Reproduced with permission from VCH Publishers, New York, NY. [From Pope, J.M., 1992. Applications or chemical shift microscopy to non-invasive histochemistry of plant materials. In *Magnetic Resonance Microscopy*, B. Blümich, and W. Kuhn (Eds.), VCH, New York.]

stuffs, and imports must be tested for exposure to ionizing radiation.

4. Evaluation of radiation dose and efficiency of irradiation is needed where irradiation is permitted.

5. Antioxidant and prooxidant activity are accompanied by transient (and sometimes permanent) production of free radicals, and understanding these processes is critical in research on natural and synthetic antioxidants used to stabilize food.

30.7.1.2 Origins of Free Radicals and Paramagnetic Ions

Situations in which free radicals and paramagnetic ions are important in foods are listed in Table 30-6 (molecules in a triplet state are not considered here). Free radicals are molecules containing an unpaired electron, e.g., carbon atom radicals in irradiated sugars. The radical must exist long enough to be detected. Most free radicals are unstable due to their chemical reactivity. Some are "scavenged" by naturally occurring compounds in foods, such as Vitamin E (α-tocopherol) and flavanoids. They generally survive longer,

the drier and more solid the material. Sometimes they may be chemically trapped (spin-trapping) long enough to be detected.

The origins of free radicals in foods are: (1) those produced by irradiation with γ-rays (as used in food sterilization) or other ionizing radiation, and (2) those produced by chemical reactions occurring naturally during ageing of food (e.g., lipid oxidation); during processing (heating, grinding, drying, freezing, etc.); during addition of antioxidants or prooxidants; or during browning (apples), greening (potatoes), reddening (meats), and other coloration reactions.

Paramagnetic ions occur naturally in food, including manganese (Mn), iron (Fe), and copper (Cu). These may occur naturally in an ESR-detectable state, but sometimes they must be brought to an appropriate oxidation state to be made ESR-detectable.

It is possible to synthesize stable free radicals for use as molecular mobility probes. They can be solubilized in oil or water, or attached covalently to specific food components such as proteins, polysaccharides, or lipids. They then can be incorporated into a food material, and used to measure changes in molecular motion that occur during gelation, adsorption, partitioning between oil and water, changes in membrane fluidity (important in oxidative stability of produce and muscle foods), protein denaturation, and other molecular processes. A probe in a liquidlike state produces narrower, better-resolved splitting patterns, while one in a solidlike state produces broader lines, up to a limit, past which further reduction in motion produces no further change in line shape.

30.7.2 Methods and Instrumentation

30.7.2.1 Bench-top ESR Spectrometer

Analytical ESR measurements on food are usually done on benchtop analyzers (schematic, Fig. 30-23) by the field sweep CW method using an electromagnet. Electron precession frequencies $\gamma B_0 / 2\pi$ are 9 GHz (gigahertz) for magnet field strengths of about 0.3 Tesla. Nine GHz corresponds to 3 cm wavelength. This is the X-band of the microwave region of the electromagnetic spectrum.

The resonance field for X-band ESR is the magnetic part of the electromagnetic radiation from a microwave source, usually a klystron or a gunn generator. The sample is contained in the resonator cavity between the poles of the magnet. During the field sweep, resonance perturbs the microwave power level in the cavity. A phase-sensitive detector picks up the signal, which is then displayed on a chart or video monitor. For sensitivity reasons, a method called field modulation often is applied. The result is that the first derivative of the ESR spectrum, dS/dB, is displayed, where S is the ESR

30-6
table   ESR Analytes In Food Materials

Source	Materials in Which Analyte Is Found
Free radicals produced during food irradiation	Meats containing bone Fish bone Spices Nuts Shellfish shells Cereal grains Frozen foods Carbohydrates (sugars, gums) Fats and oils Fresh and dried fruits Fresh and dried vegetables
Free radicals produced in food processes	Lipid oxidation (meats, fish, vegetable oil, oilseeds, orange juice essence, etc.) Oxidation of natural polyphenolic compounds (teas, apples, etc.) Coffee aging, coffee extraction During Vitamin E activity in oils Beer staling Unsaturated fat oxidation of protein Browning reactions (many foods) Greening reactions (potato) Blueing reactions Reddening reactions (meats)
Paramagnetic ions	Copper in soy sauce and various foods Mn in various foods Cu in various foods Fe in various foods
Added free radical probes used to report molecular mobility and binding	Starch Gluten Whey protein concentrates Oil and water contents Carbohydrate glass transition Protein glass transition Membrane fluidity
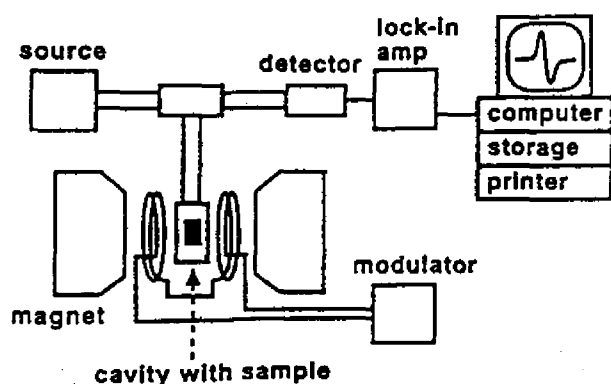
30-23
figure   Schematic diagram of an ESR spectrometer for analysis of free radicals and paramagnetic ions in food materials.

signal and B is the field strength (see the example in section 30.7.3).

30.7.2.2 Samples

Samples for ESR analysis can be nearly anything. Volumes are generally small, typically 0.1 cm^3 (or 20 mg of a powder) but larger in bench-top ESR instruments. High quality sample tubes (quartz) are usually needed. Sample shape and position affect results. Since water absorbs microwaves efficiently, detection efficiency is better the lower the H_2O content of the sample.

30.7.2.3 Analysis of ESR Data

A free electron has a spin quantum number of 1/2. Thus there are two energy levels in a magnetic field,

one transition between them, and therefore just one line in the ESR spectrum. As in NMR, the electron spin magnetic moment interacts with the local magnetic field. However, instead of expressing this as the chemical shift, in ESR the resonance condition is expressed as

$$hv = g\mu_B B \qquad [21]$$

where:

μ_B = Bohr magneton
g = electron g-factor of the paramagnetic species.

The position of the resonance in the ESR spectrum is usually given in units of the magnetic field instead of frequency, e.g., 320 milliTesla (mT). From this, the g-factor for the species can be calculated using Equation [20] and the fact that $h/\mu_B = 71.4448$ mT/GHz.

A free electron has a g-factor g_e value of 2.0023. Radicals and ions in foods have values that are different from this, and this sometimes helps identify the ESR-active species. Another aid to assignment is the fact that ESR resonances often are split (in ways that depend on molecular structure) via spin-spin interactions with other electrons or nearby magnetic nuclei. Mn^{2+}, for example, often found in seafood, often gives a six-line ESR resonance.

Intensity of the ESR signal is measured either as peak height or peak area over a certain range of the ESR spectrum. Results then can be expressed as ESR signal intensity per unit weight of sample. This and the principles for assignment (previous paragraph) form the basis for quantitative analysis of food materials by ESR.

If several ESR-active species are present, their signals will superimpose. Irradiation of food with γ-rays often will produce free radicals, and thus an ESR response appears where there was none before, or irradiation may add a new kind of signal to a naturally occurring one in the ESR spectrum.

30.7.3 Example: ESR Analysis of Irradiated Chicken (Fig. 30-24)

Fresh chicken was irradiated with γ-rays from a cobalt-60 source. Freeze-dried chicken bone powder isolated from irradiated chicken was examined in a Bruker ESP 300 ESR spectrometer with TE104 cavity. This is an electromagnet, X-band ESR instrument. Gamma irradiation induces formation of CO_2^- radicals in crystalline hydroxyapatite regions in bone. Intensities were normalized by the sample weight. It is clear that ESR signal intensity per gram of bone powder is proportional to irradiation dose. The irradiation dose for an unknown thus could be determined by measuring the ESR signal and interpolating from the dose–response curve.

349.5 mT

10 kGy
7.5 kGy
5.0 kGy
2.5 kGy

1 mT
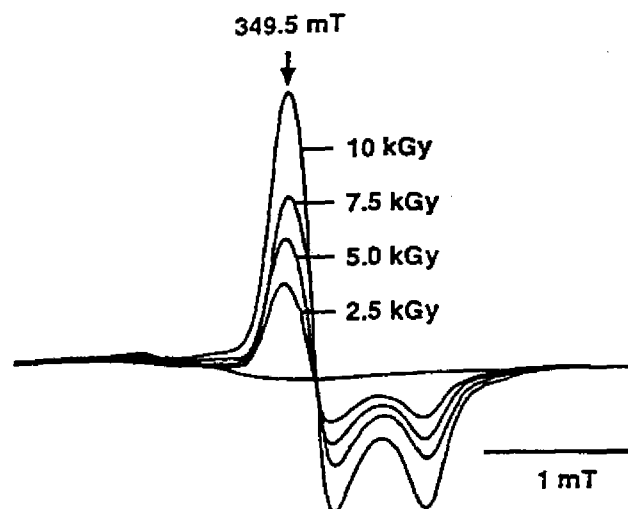
30-24 figure Effect of irradiation dose on ESR signal from freeze-dried chicken bone powder obtained from γ-irradiated chicken. (Reproduced with permission from Bruker Instruments, Inc., from Gray, R. and Stevenson, M.H. 1992. The use of ESR spectroscopy for the identification of irradiated food. In *Bruker Report*, 1991-1992, Bruker Instruments, Billerica, MA.)

30.8 SUMMARY

The principles of magnetic resonance are the same for NMR and ESR. A sample magnetization can be manipulated to produce a detectable signal. Due to the sensitivity of nuclei and electrons to molecular structure and motion, it is possible to extract chemical (compositional, reactions) and physical information (solidity, liquidity, diffusion, flow) from food samples. Instrumentation involves a magnet to polarize the spins; a transmitter of an oscillating magnetic field; a probe to hold the sample and permit excitation and detection; a receiver; and a data system. It is often possible to control sample environment to correspond to functional conditions. Robotics and connections to laboratory information management systems are implemented in commercial instruments. Magnetic resonance methods are essentially nondestructive, except for preparing a sample that fits the probe. Potential issues are sensitivity and selectivity. Problems with magnetic resonance analysis due to expense and complexity are very quickly yielding to new technology and education of users. Magnetic resonance devices serve in food research and analytical labs, in quality control labs, and as sensors in food processing operations.

30.9 STUDY QUESTIONS

1. Why is the sample immersed into a magnetic field?
2. What is the effect on nuclear precession of increasing the field strength?

3. What is the effect on the magnetization M, of applying an oscillating magnetic field $B_1 cos(\omega t)$, that is on-resonance (ω = Larmor frequency ω_L), and perpendicular to the static field, for a shorttime t_p?

4. In pulse NMR, why do we apply a 90° pulse?

5. What is the free induction decay? What must you do to get the NMR spectrum from the free induction decay?

6. What is transverse relaxation? Describe two kinds of T_2 measurements. Why does transverse relaxation happen?

7. What is longitudinal relaxation? Describe a measurement of T_1. Why does longitudinal relaxation happen?

8. Why is it possible to distinguish all the different atoms in a molecule in a high resolution NMR spectrum?

9. How are the NMR signals from rigid crystalline, viscous, and fluid phases different? Why are they different? What kind of relaxometry measurement can obtain all three signals in a single measurement?

10. What are three characteristics of a resonance in an NMR spectrum that are useful for chemical analysis?

11. List the four major types of analytical NMR. For each, give two types of measurements. For each type of measurement, name one application to food analysis.

12. What are three reasons to measure free radical activity in foods?

13. Why do you expect to find an ESR signal for just about every food material? Why are they not always evident?

14. In NMR, a signal is primarily identified by its resonance frequency or chemical shift. What is the corresponding property in ESR?

15. Draw the chemical structure of glucose. (a) How many chemically inequivalent carbons are there? (b) How many magnetically inequivalent carbons? (c) Carbon resonances are split by J-coupling to bonded protons. Assume that you use low power proton decoupling to avoid this. How many resonances are there in the solution spectrum? (d) What is the ratio of peak intensities? (e) If an aliphatic carbon is more shielded by local electrons than an alcoholic carbon atom, then which will resonate at a higher frequency, the C-6 carbon or the C-2 carbon?

16. Draw a triacylglycerol molecule with three C-16 fatty acyl chains. Give each chain a double bond at the (9,10) position (the carbonyl carbon is position 1). (a) On this picture, identify the following chemical functional groups: methyl, methylene, methene, ester, glyceryl. (b) Protons of each type resonate in different regions of the NMR spectrum. What are the relative areas in the spectrum of this pure compound in a liquid state? [Hint: How many hydrogens (and therefore ^1H nuclei) are in each group?]. (c) Using NMR, how could you estimate the unsaturated/saturated ratio for fatty acids in an unknown fat?

30.10 PRACTICE PROBLEMS

1. **Quality assessment.** Pick a specific snack food from the following general groups: fresh fruit, nuts, cookies, chips (any kind), cured meat, cheese. You are in charge of quality control for that product. You already assessed textural quality by making instrumental measurements, but you want to try non-destructive evaluation. In a flash of inspiration, NMR comes to mind. To decide what NMR measurements to make, you will have to answer the following questions: (a) What precisely are the *sensory* textural qualities that are important to product acceptance and liking? (b) What are the *instrumental* textural measurements you would normally make in quality control? (c) Since structure and composition determine texture, describe the structure at the visible and microscopic levels. (d) What are the solid, semisolid, and liquid phases that exist in this food? What are their relative amounts (in grams per 100 grams of product)? (e) What NMR measurements would you make that should correlate well with instrumental and sensory texture assessment? (f) Other kinds of sensory qualities, like flavor, are harder to assess by NMR. What are some complementary food analytical methods for total quality assessment for this particular food?

2. **Water mobility in bread.** Scores for perceived moistness and tenderness are lower (compared to a standard recipe) for bread made with flour from a new wheat variety. Both breads have 38% moisture by analysis. From the clue about moistness, you think it has something to do with the *mobile* water. You decide to estimate water mobility in terms of the water proton transverse relaxation time T_2. You design a CPMG measurement to detect the most mobile water, by using a value of t = 5 msec for the delay between the 90° and 180° pulses. You collect ten successive echoes. The intensities are tabulated below. (a) From these data, obtain a value for the water proton T_2. (b) Compare this value with that for pure water, $T_2 = 3.0$ sec. (c) Propose an explanation of why they are so different.

n	M(t)	n	M(t)
1	709	6	125
2	522	7	98.2
3	368	8	69.5
4	268	9	47.3
5	182	10	36.5

3. **Phases in frozen fruit.** The total proton free induction signal from a sample of frozen fruit at home freezer temperature (-18°C) was fit to a sum of three decays. The type, amplitude, and decay time T_2^* are given in the following table.

Type of decay	Amplitude	T_2^*/sec
rapid Gaussian	529	8.0×10^{-6}
intermediate exponential	189	9.0×10^{-5}
slow exponential	37.8	1.6×10^{-3}

(a) From the amplitudes, calculate the relative amount of each decay component. (b) From the decay times T_2^*, calculate the line widths (in kHz) that would be observed if you transformed the FID to a spectrum. (c) Propose the origin of each signal. [Hints: (1) The fruit sample is 90% water, 6% sugars, and about 3% cell wall material. (2) Upon freezing, ice crystallites form within vacuoles in cells, leaving a freeze-concentrated mixture of water and solutes between them. Concentrated mixtures can get

highly viscous as temperature goes down. (3) Some water in foods is resistant to freezing. The unfreezable amount is proportional to the macromolecular content of the sample.] (d) Why do you think that deteriorative reactions that affect aroma, color, and nutrient content of frozen foods can occur at home freezer temperature?

4. Brining of cheese. You are working with food engineers to optimize the brining step in Cheddar cheese manufacture. The engineers need data for their model for migration of salt into the cheese slabs. You design a magnetic resonance imaging measurement to get the data. (a) Draw a cross-section of a cheese slab. Indicate the position of the advancing salt boundary at different times of brining. (b) What magnetic nuclei are candidates for imaging this process? Which do you prefer and why? (c) Would you use 1-D, 2-D, or 3-D imaging? Why? (d) What do you think is the better basis for contrast: (1) spin density (proportional to concentration of the observed species), or (2) spin relaxation (proportional to rotational mobility and local interactions with other molecules)? Justify your choice.

5. Food irradiation. Using the data in Fig. 30-24: (a) Construct the dose-response curve for chicken bone powder from irradiated chicken. [Hint: Measure the major peak height in mm, then divide all heights by the largest value.] (b) You make an ESR measurement on chicken bone powder from a sample of unknown radiation history, obtaining a value of 0.55. What was the probable radiation dose? (c) Is the sensitivity of the ESR measurement higher at lower or higher radiation doses? (d) Why are measurements made on powder rather than meat?

6. Composition of intact fruit tissue. By analyzing integrals of various peaks in the strawberry spectrum in Fig. 30-17, the following results were obtained:

Solute	Content +/-SD (g/100g tissue)
sucrose	1.50 +/-0.20
glucose	0.90 +/-0.12
fructose	2.99 +/-0.39
citrate	0.43

(a) Calculate the molar ratios of these solutes (sucrose MW = 342.3, glucose 180.16, fructose 180.16, citrate 191.12). (b) How are the molar ratios related to perceived sweetness? (c) The spectrum in Fig. 30-17 is still crowded. Specify two ways to relieve the crowding and explain briefly why each will work.

7. Fat droplet size distribution in cheese and cream. Pulsed field gradient NMR was applied to samples of Cheddar cheese, Swiss cheese, and cream. Curves like those shown in Fig. 30-20 were obtained. The data were fit to Equation [20] to obtain the mean droplet radius R_0, and the width of the distribution expressed as its standard deviation. The results are given in the following table.

Sample	R_0/nm	SD/nm
Cheddar cheese	2100	1130
Swiss cheese	2650	1100
cream	1650	550

(a) Sketch these distributions. [Hint: Assume normal distribution (bell-shaped), and allow all curves to have the same height in your sketch.] (b) List the samples in descending order of mean particle radius. (c) List the samples in descending order of the width of the distribution. (d) Why do you think the cheese droplet size distributions are so much wider than the cream distribution? (e) Sensory qualities of appearance, stability, texture, and flavor of cheese might be affected by the mean radius and distribution width. Why?

8. NMR of cinnamon roll. A cinnamon roll has the moisture content of white pan bread; shortening and sugar were added to the dough; and the smear is made from margarine, water, and brown sugar. Much to your surprise, the magic angle spinning proton NMR spectrum taken at room temperature reveals resolvable peaks in the spectral regions expected for triacylglycerol protons (Study Question 16), a cluster of peaks in the carbohydrate region (something like those in Fig. 30-17), and a sizable water peak. (a) What can you say about the identity and motion of the molecules producing these resonances? (b) Both margarine and shortening are semisolid fats. In the first approximation, how much of the total fat do you expect to be represented in the high resolution spectrum? (c) When you taste the roll, you notice sugar crystals. If you know the total sugar, and can measure the mobile sugar from the proton spectrum, how much of the sugar is crystalline? (d) If you wanted to "see" the crystalline fractions of fats and sugars, what high resolution NMR method would you use?

9. MRI of cinnamon roll. Conventional MRI often uses a spin echo pulse sequence (90° - TE - 180° - echo), and the voltage at the top of the echo is recorded. The value of TE can be selected by the operator. If TE is short (compared to liquid transverse relaxation), then liquid components with T_2s from short to long are detected. If TE is long, then liquid components with long T_2s are favored. To prepare to do some MRI measurements of cinnamon rolls, you first estimate T_2 by the line width method for all peaks in the magic angle spinning proton NMR spectrum for the cinnamon roll (see Problem 8), and get the following values: water, 10 msec; sugar, 50 msec; fatty acyl, 200 msec. Now you are ready to do MRI. (a) How would you discriminate against the water signal? What would you then be imaging? (b) How would you discriminate against the water and sugar signals? What would you then be imaging?

Answers

1. Quality assessment. It is up to the instructor and students to choose a snack food and answer questions (a) sensory textural qualities; (b) instrumental textural measurements (see Chapter 34 in this book); (c) description of structure and composition at the visible and microscopic levels; (d) the solid, semisolid, and liquid phases that exist in this food, and their relative amounts. For (e), con-

sult Tables 30-3, 30-4, and 30-5 for NMR measurements that should correlate with texture assessment. (f) Complementary analytical methods for quality assessment include quantitative sensory evaluation, gas chromatography and high pressure liquid chromatography to identify flavors and aromas, differential scanning calorimetry to measure crystallinity or glass transition, and so on.

2. Water mobility in bread. Students should refer to section 30.2.11 for the CPMG equations, and Fig. 30-9 for the CPMG pulse sequence. (a) Create a table with four columns: n (given), t = 2τn (calculate from n and τ = 5 msec), M(t) (given), and lnM(t) (calculate). Draw the graph y = lnM(t) with values of the ordinate from 3.0 to 7.0; x = t = 2τn/msec, with the range on the abscissa from 0 to 100. Draw the best straight line through the points. Select two widely separated points on the line, and calculate the slope [M(t1) - M(t2)]/(t1 - t2)]. From the fact that the slope = -1/T_2 calculate T_2. Answer: 30 msec. (b) This is two order of magnitude shorter than T_2 of pure water. (c) Possible reasons are: (1) Water motion is somewhat restricted because much of it is confined to hydration layers on starch and protein. (2) Water molecules that hydrogen-bond to strong donors and acceptors on the macromolecular surfaces are less mobile than water molecules hydrogen-bonded to each other. (3) Water protons may exchange with labile macromolecular protons: starch hydroxyl -OH; protein amine -NH_3^+ (lys), -NH-CNH_2^+)-NH_2 (arg); protein carbonyl -COO^- (glu, asp); protein amide -CONH (peptide) and -$CONH_2$ (gln, asn). Exchange shortens the water proton T_2.

3. Phases in frozen fruit. Students should refer to section 30.2.12 and Fig. 30-10 for the free induction decay, and to section 30.2.11 for the equation to calculate line width from T_2 (a) Complete the table: add a column for (A/ΣA) ×100%, and one for line width $\Delta v_{1/2} = 1/\pi T_2^*$/Hz. (a) The relative amounts are 70%, 25%, and 5%. (b) The line widths are 39.8 kHz, 3.54 kHz, and 0.199 kHz. (c) The 70% signal is due to water protons in ice. The 25% signal is due to the freeze-concentrated phase and contains protons from sugar and water. The 5% signal is the liquid that hydrates cellular organelles, mainly cell walls, and contains protons from water and maybe some sugars. (d) Deteriorative reactions occur because reactants may diffuse through the hydration phase. Enzymes may also be active in this phase. Functional groups in macromolecules are more accessible for reaction when they are hydrated.

Additional questions for students: How would this kind of analysis be useful for understanding stability and sensory properties of ice cream? For quality assurance in frozen food processing plants?

4. Brining of cheese. Students should refer to section 30.6 for general imaging, Table 30-1 for relative sensitivities of nuclei, and Table 30-5 for types of MRI contrast. (a) The cross section shows concentric rectangles, the innermost being the brine front at the latest time of observation. (b) Sodium-23 (^{23}Na) is the best choice for nucleus detected in MRI, because the species of interest is the sodium ion Na*. Chlorine-35 (^{35}Cl) is an obvious alternative. However, Table 30-5 shows that the sensitivity (relative to ^{1}H) for detection of ^{23}Na is 9.25 × 10^{-2}, whereas the sensitiv-

ity for ^{35}Cl is only 4.7 × 10^{-3}. ^{1}H would be a good choice if some property of the ^{1}H signal (line width, T_2, T_1 diffusion constant, etc.) were found to be proportional the local salt concentration. This is possible, and would require testing these different bases of contrast. Students should be given credit if they choose ^{1}H and make the point about proportionality to salt concentration. (c) 2-D imaging (image of a virtual slice) is the best because it will produce a picture just like the drawing. (d) Spin density is the preferred basis for contrast, because the pixel intensity at each location will then be directly proportional to concentration.

Additional questions for students: Name some other food processes that involve migration of a low molecular weight component into a matrix. [Answer: layered foods (e.g., pizza), candies with centers (e.g., peanut butter filled chocolate cups), filled snacks, etc.] What would a series of images taken at different times of brining look like? [Answer: Assume pixel is brighter, the higher the local ^{23}Na concentration. Then as time goes on, brightness moves toward center of slab cross section.] How would one convert this series into a mass diffusivity map? [Answer: For every pixel in the image, draw the intensity vs. time curve, and extract the time constant by fitting to a simple decay function. Then create a new image where pixel intensity at a point is equal to the time constant at the point.]

5. Food irradiation. Students should refer to section 30.7 for general ESR, section 30.7.2.3 for quantitative ESR, and Fig. 30-24 for the data. (a) Create a table with three columns: dose/kGy (given), signal height/mm (measured by student), normalized signal = signal height/max signal height (dimensionless). Draw the graph y = normalized signal, ranging from 0 to 100; x = dose/kGy, ranging from 0 to 10. Draw the best smooth curve through the points. (b) approximately 6.7 kGy (the value will depend on how the student draws the curve and their skill in graphic interpolation.) (c) Sensitivity of the measurement is greater at higher radiation doses. (d) Free radicals are more stable in dry than in wet materials.

6. Composition of intact fruit tissue. Students should refer to section 30.4 for general high resolution NMR, section 30.4.1.1 for single pulse NMR, section 30.4.3.1 for strawberry, Fig. 30-17 for strawberry, and Table 30-4 for hints on relieving spectral crowding. (a) Complete the table as shown:

Solute	Content +/-SD (g/100g tissue)	Content/ MW	Content/MW ÷ value for glucose
sucrose	1.50 +/-0.20	0.00438	0.876
glucose	0.90 +/-0.12	0.00500	1.000
fructose	2.99 +/-0.39	0.0166	3.32
citrate	0.43	0.00225	0.45

(b) Molar amounts determine sweetness, not weight percent, because taste receptors interact with single molecules. Fructose, which is sweeter than glucose or sucrose, and has a large molar excess in this particular strawberry, will dominate sweetness. (c) Select two: (1) Increase field strength B_0, then ^{1}H resonance frequency increases from 200 MHz (Fig. 30-17) to 400, 500, 600 MHz, or higher. The

separation between peaks will increase proportionately, thus relieving crowding. Sensitivity will also increase (section 30.2.17 and Fig. 30-14). (2) Use 2-D NMR (e.g. homonuclear correlation spectroscopy (COSY), but students would not be expected to know that). Resonances get spread out over two dimensions. (3) Switch to carbon-13 detection. ^{13}C has a 20-fold greater range in chemical shift, so peaks are more spread out. Also, if low power proton decoupling is used, there will be only one resonance per carbon atom, compared to multiplets for every hydrogen atom as in ^{1}H NMR.

Additional questions for students: How could high resolution NMR analysis be applied to quality control for sweetness perception? [Answer: It tells geneticist, grower, and processor the target sugar mixture.] How could this kind of analysis be used in processing that directly affects liquid-phase composition? [Answer: thermal processing, enzyme processing, fermentation, etc.]. How could this kind of analysis be used for quality assessment? [Answer: readiness for processing, storage, transport, or consumption of fresh fruit and vegetables.]

7. Fat droplet size distribution in cheese and cream. Students should refer to section 30.5 for general PFG NMR, and especially section 30.5.3, Fig. 30-20, and Equation [20] for droplet size distribution analysis. (a) Draw three curves, each bell-shaped. The range on the abscissa is from 0 to 5000 nm. The maximum of each curve is right above its value for R_0 on the abscissa. The width of each curve at half height is approximately equal to two times the SD. The curve for cream is to the left of the others, and is narrower. The curves for the cheeses are very broad, and overlap considerably. (b) Swiss > Cheddar > cream. (c) Cheddar > Swiss > cream. (d) Droplets probably aggregate during cheesemaking and ageing. The emulsified droplets may become less stable due to the action of proteases on proteins that stabilize the emulsion surface. (e) Appearance: more particles, especially those with sizes near the wavelength of light, means more opaque, and lighter color. Texture: hard to predict, but smaller particles could mean stronger protein network architecture, thus greater hardness, less fracture. Stability: oiling-off occurs in natural cheeses because fat droplets are larger, and have greater tendency to break down into free fat and escape at a cut surface. Flavor: smaller droplets could mean less flavor and aroma impact, at least for the oil-soluble stimulants, because not as many droplets are broken down upon cutting or chewing. Comment: While it might have confused the students to include the experimental uncertainties, the standard deviations (SD) for measurement (as distinguished from the SD of the distribution, a parameter of the curve fit) for reported values of R_0 and SD were actually very small. Thus the PFG NMR measurement of droplet size distribution has good precision, and differences between these distributions are quite real despite their broadness.

8. (a) The motion of the molecules producing these resonances must be very fast; thus the molecules are in a liquid-like state, and are probably located in physically identifiable liquid domains. (b) The fraction of total fat represented in the high resolution spectrum would be equal to (1- $SFC_{margarine}$) × margarine content, plus (1 -

$SFC_{shortening}$) × shortening content, divided by the sum of margarine and shortening. SFC of course is the solid fat content measured by NMR on the ingredients. (c) Crystalline sugar = total sugar (from the formulation) minus the mobile sugar (from NMR). (d) To "see" the crystalline fractions of fats and sugars, you would use high resolution solids carbon-13 NMR (see Table 30-4).

9. MRI of cinnamon roll. (a) You discriminate against the water signal by using a TE > 30 msecs. You then would be imaging mobile sugar and fat. (b) You discriminate against water and sugar by using a TE > 150 msec. You then would be imaging mobile fat only. A TE series can provide very interesting results when comparing samples in which a principle component has a different molecular state in one sample compared to the other. Similarly, a series would reveal much about full-fat versus fat-replaced rolls.

30.11 RESOURCE MATERIALS

30.11.1 Introductory NMR and ESR

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Martin, M.L., Delpeuch, J.J., and Martin, G.J. 1980. *Practical NMR Spectroscopy*. Heyden & Son, Philadelphia.

Knowles, P.F. 1976. *Magnetic Resonance of Biomolecules, an Introduction to the Theory and Practice of NMR and ESR in Biological Systems*. John Wiley & Sons, New York.

30.11.2 Books and Reviews on Magnetic Resonance in Food Science

(Multiple articles and authors). 1992. Special issue: Applications of nuclear magnetic resonance techniques in food research. *Trends in Food Science and Technology* 3:177–250.

Webb, G.A., Belton, P.S., and McCarthy, M.J., (eds.). 1995. *Annual Reports on NMR Spectroscopy*, Vol. 31. Academic Press, San Diego, CA. (Special issue on food).

Belton, P.S., Delgadillo, I., Gil, A.M., and Webb, G.A., (Eds.). 1995. *Magnetic Resonance in Food Science*. The Royal Society of Chemistry, Cambridge, England.

Watanabe, H., Fukuoka, M., and Watanabe, T. 1995. Recent advances in characterization of foods using nuclear magnetic resonance (NMR), in *Characterization of Food: Emerging Methods*, A.G. Gaonkar (Ed.). Elsevier, New York.

Hanna, G.M., and Specchio, J.J. 1995. Selected NMR applications in food regulatory analysis. *Food Testing and Analysis*, October/November, 43–46.

30.11.3 Relaxometry (Mobility and Phases)

Gambhir, P.N. 1992. Applications of low-resolution pulsed NMR to the determination of oil and moisture in oilseeds. *Trends in Food Science and Technology* 3:191–196.

Gribnau, M.C.M. 1992. Determination of solid/liquid ratios of fats and oils by low-resolution pulsed NMR. *Trends in Food Science and Technology* 3:186–190.

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part IV

Chromatography

31 chapter

Basic Principles of Chromatography

Mary Ann Rounds and S. Suzanne Nielsen

31.1 INTRODUCTION

The impact of chromatography has been very great on all areas of analysis and, therefore, on the progress of science in general. Chromatography differs from other methods of separation (e.g., fractional distillation, in which more or less the same operations and apparatus are always employed) in that a wide variety of materials, equipment, and techniques can be used. We have chosen to approach this extremely broad and complex field by first describing the general principles of extraction as a basis for understanding chromatography. This chapter will focus on liquid chromatography, subdivided as shown in Fig. 31-1. The related technique of supercritical fluid chromatography is described briefly in section 31.3.4.4. In view of their widespread use, gas chromatography and high performance liquid chromatography are the topics of separate chapters.

31.2 EXTRACTION

In its simplest form, extraction refers to the transfer of a solute from one liquid phase to another. Extraction in myriad forms is integral to food analysis—whether used for preliminary sample cleanup, concentration of the component of interest, or as the actual means of analysis. Extractions may be carried out by means of batch, continuous, or countercurrent processes. (Various extraction procedures are discussed in detail in other chapters: traditional solvent extraction in Chapters 13, 20, and 33; accelerated solvent extraction in Chapters 13 and 20; solid-phase extraction in Chapter 20 and 33; and solid-phase microextraction and microwave-assisted solvent extraction in Chapter 20.)
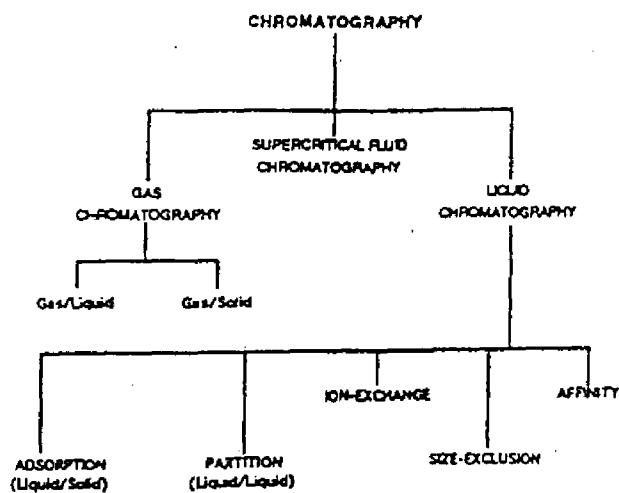
CHROMATOGRAPHY

A scheme for subdividing the field of chromatography.

31.2.1 Batch Extraction

In batch extraction the solute is extracted from one solvent by shaking it with a second, immiscible solvent. The solute partitions, or distributes, itself between the two phases and, when equilibrium has been reached, the partition coefficient, K, is a constant.

$$K = \frac{\text{concentration of solute in Phase 1}}{\text{concentration of solute in Phase 2}} \quad [1]$$

The phases are allowed to separate, and the layer containing the desired constituent is removed, for example, in a separatory funnel. In batch extraction, it is often difficult to obtain a clean separation of phases, owing to emulsion formation. Moreover, partition implies that a single extraction is usually incomplete.

31.2.2 Continuous Extraction

Continuous liquid–liquid extraction requires special apparatus, but is more efficient than batch separation. One example is the use of a Soxhlet extractor for extracting materials from solids. Solvent is recycled so that the solid is repeatedly extracted with fresh solvent. Other pieces of equipment have been designed for the continuous extraction of substances from liquids. Different extractors are used, depending on whether the nonaqueous solvent is heavier or lighter than water.

31.2.3 Countercurrent Extraction

Countercurrent distribution refers to a serial extraction process. It separates two or more solutes with different partition coefficients from each other by a series of partitions between two immiscible liquid phases. (Countercurrent distribution was once an important separation technique used by lipid chemists.) Liquid–liquid partition chromatography is a direct extension of countercurrent extraction; its modern version is known as countercurrent chromatography.

31.3 CHROMATOGRAPHY

31.3.1 Historical Perspective

Modern chromatography originated in the late nineteenth and early twentieth centuries from independent work by David T. Day, a distinguished American geologist and mining engineer, and Mikhail Tsvet, a Russian botanist. Day developed procedures for fractionating crude petroleum by passing it through Fuller's earth; Tsvet used a column packed with chalk to separate leaf pigments into colored bands. Because Tsvet recognized and correctly interpreted chromatographic

processes and named the phenomenon chromatography, he is generally credited with its discovery.

After languishing in oblivion for years, chromatography began to evolve in the 1940s due to the development of column partition chromatography by Martin and Synge and the invention of paper chromatography. The first publication on gas chromatography (GC) appeared in 1952. By the late 1960s, GC, because of its importance to the petroleum industry, had developed into a sophisticated instrumental technique. Since early applications in the mid-1960s, high performance liquid chromatography (HPLC), profiting from the theoretical and instrumental advances of GC, has extended the area of liquid chromatography into an equally sophisticated and useful method. Supercritical-fluid chromatography (SFC), first demonstrated in 1962, is finally gaining popularity. Modern chromatographic techniques, including automated systems, are widely utilized in the characterization and quality control of food raw materials and food products.

31.3.2 General Terminology

Chromatography is a general term applied to a wide variety of separation techniques based on the partitioning or distribution of a sample (solute) between a moving or mobile phase and a fixed or stationary phase. (Chromatography may be viewed as a series of equilibrations between the mobile and stationary phases.) The relative interaction of a solute with these two phases is described by the partition (K) or distribution (D) coefficient (ratio of concentration of solute in stationary phase to concentration of solute in mobile phase). The mobile phase may be either a gas (GC) or liquid (LC) or a supercritical fluid. The stationary phase may be a liquid or, more usually, a solid. The field of chromatography can be subdivided or organized in several different ways, according to the physicochemical principles involved in the separation or according to the various techniques applied. Table 31-1 summarizes some of the chromatographic procedures or methods that have been developed on the basis of different mobile–stationary phase combinations. Inasmuch as the nature of interactions between solute molecules and the mobile or stationary phases differ, these methods have the ability to separate different kinds of molecules. (The reader is urged to review Table 31-1 again after having read this chapter.)

As previously stated, this chapter is devoted primarily to the area of liquid chromatography (as performed at atmospheric pressure). Gas chromatography is covered in Chapter 33 and HPLC is the subject of Chapter 32. Supercritical fluid chromatography (SFC) refers to chromatography performed at pressures and temperatures above the critical values of the mobile phase; a supercritical fluid (or compressed gas)

is neither a liquid nor a typical gas. Consequently, SFC complements both GC and HPLC and can overcome some of the problems associated with each. An overview of this technique is provided in section 31.3.4.4.

31.3.3 Physicochemical Principles of Separation

To provide the reader with a broad understanding and better perspective of the field, we have chosen to describe first the physicochemical principles (illustrated in Fig. 31-2) that underlie liquid chromatographic separations, regardless of the specific techniques applied. Although it is more convenient to describe each of these phenomena separately, it must be emphasized that more than one mechanism may be involved in a given fractionation. For example, many cases of partition chromatography also involve adsorption.

31.3.3.1 Adsorption (Liquid–Solid) Chromatography

Adsorption chromatography is the oldest form of chromatography, having been used by Tsvet in 1903 in the experiments that spawned modern chromatography. In this chromatographic mode, the stationary phase is a finely divided solid (to maximize the surface area), and the mobile phase may be either a gas or a liquid. (Gas–solid adsorption chromatography is discussed in Chapter 33.) The stationary phase (adsorbent) is chosen to permit differential interaction with the components of the sample to be resolved. The intermolecular forces thought to be primarily responsible for chromatographic adsorption include:

1. Van der Waals forces
2. Electrostatic forces
3. Hydrogen bonds
4. Hydrophobic interactions

Sites available for interaction with any given substance are heterogeneous. Binding sites with greater affinities, the most active sites, tend to be populated first, so that additional solutes are less firmly bound. The net result is that adsorption is a concentration-dependent process, and the adsorption coefficient is not a constant (in contrast to the partition coefficient). Sample loads exceeding the adsorptive capacity of the stationary phase will result in relatively poor separation.

Classical adsorption chromatography utilizes silica (slightly acidic), alumina (slightly basic), charcoal (nonpolar), and a few other materials as the stationary phase. Both silica and alumina possess surface hy-

31-2
table   Characteristics of Different Chromatographic Methods

Method	Mobile/Stationary Phase	Retention Varies with
Gas–liquid chromatography	Gas/liquid	Molecular size/polarity
Gas–solid chromatography	Gas/solid	Molecular size/polarity
Supercritical-fluid chromatography	Supercritical fluid/solid	Molecular size/polarity
Reversed-phase chromatography	Polar liquid/nonpolar liquid or solid	Molecular size/polarity
Normal-phase chromatography	Less polar liquid/more polar liquid or solid	Molecular size/polarity
Ion-exchange chromatography	Polar liquid/ionic solid	Molecular charge
Size-exclusion chromatography	Liquid/solid	Molecular size
Hydrophobic-interaction chromatography	Polar liquid/nonpolar liquid or solid	Molecular size/polarity
Affinity chromatography	Water/binding sites	Specific structure

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droxyl groups, and Lewis acid-type interactions determine their adsorption characteristics. The elution order of compounds from these adsorptive stationary phases can often be predicted on the basis of their relative polarities (Table 31-2). Compounds with the most polar functional groups are retained most strongly on polar adsorbents and, therefore, are eluted last. Nonpolar solutes are less well retained on polar adsorbents and are eluted first.

One model proposed to explain the mechanism of liquid–solid chromatography is that solute and solvent molecules are competing for active sites on the adsorbent. Thus, as relative adsorption of the mobile phase increases, adsorption of the solute must decrease. Solvents can be rated in order of their strength of adsorption on a particular adsorbent, such as silica. Such a solvent strength (or polarity) scale is called an eluotropic series. Table 31-3 is an example of such a series for alumina. (Silica has a similar rank ordering.) Eluotropic series provide the chromatographer with a way to modulate interaction between solutes and the stationary phase. Once an adsorbent has been chosen, solvents can be selected from the eluotropic series for that adsorbent. Mobile phase polarity can be increased (often by admixture of more polar solvents) until elution of the compound(s) of interest has been achieved.

Adsorption chromatography separates aromatic or aliphatic nonpolar compounds, such as lipids, primarily according to the type and number of functional groups present. The labile, fat-soluble chlorophyll and carotenoid pigments from plants have been studied extensively by adsorption chromatography (Tsvet's original experiment) utilizing columns. Adsorption chromatography also has been used for the analysis of fat-soluble vitamins. Frequently, it is used as a batch procedure for removal of impurities from samples prior to other analyses. For example, disposable solid-phase extraction cartridges (see Chapter 33) containing silica have been used for food analyses, such as lipids

in soybean oil, carotenoids in citrus fruit, and vitamin E in grain.

31.3.3.2 Partition (Liquid–Liquid) Chromatography

31.3.3.2.1 Introduction In 1941, Martin and Synge undertook an investigation of the amino acid composition of wool, using a countercurrent extractor of 40 tubes with chloroform and water flowing in opposite directions. The efficiency of the extraction process was improved enormously when a column of finely divided inert support material was used to hold one liquid phase (stationary phase) immobile, while the second liquid, an immiscible solvent (mobile phase), flowed over it, thus providing intimate contact between the two phases. Solutes partitioned between the two liquid phases according to their partition coefficients, hence the name partition chromatography.

A partition system is manipulated by changing the nature of the two liquid phases, usually by combination of solvents or pH adjustment of buffers. Often, the more polar of the two liquids is held stationary on the inert support and the less polar solvent is used to elute the sample components (normal-phase chromatography). Reversal of this arrangement, using a nonpolar stationary phase and a polar mobile phase, has come to be known as reversed-phase chromatography (see section 31.3.3.2.3).

Polar hydrophilic substances, such as amino acids, carbohydrates, and water-soluble plant pigments, are separable by normal-phase partition chromatography. Lipophilic compounds, such as lipids and fat-soluble pigments, may be resolved with reversed-phase systems. Liquid–liquid partition chromatography has been invaluable to carbohydrate chemistry. Column chromatography on finely divided cellulose has been used extensively in preparative chromatography of sugars and their derivatives. Paper chromatography

Adsorption chromatography

- Solute adsorbed on surface of stationary phase

Partition chromatography

- Solute dissolved in liquid phase coated on surface of solid support

Ion-exchange chromatography

- Mobile anions held near cations that are covalently attached to stationary phase
- Anion-exchange resin, since only anions can be attracted to it

Molecular exclusion chromatography

- Large molecules are excluded
- Small molecules penetrate pores of particles

Affinity chromatography

- One kind of molecule in complex mixture becomes attached to molecule that is covalently bound to stationary phase
- All other molecules simply wash through

31-2 figure Physicochemical principles of chromatography. (From *Quantitative Chemical Analysis*, 4th ed. by D.C. Harris. © 1995 by W.H. Freeman & Co. Used with permission.)

(section 31.3.4.1) is a simple method for distinguishing between various forms of sugars or phenolic compounds present in foods.

31.3.3.2.2 Coated Supports In its simplest form, the stationary phase for partition chromatography consists of a liquid coating on a solid matrix. The solid support

should be as inert as possible and have a large surface area in order to maximize the amount of liquid held. Some examples of solid supports that have been used are silica, starch, cellulose powder, and glass beads. All are capable of holding a thin film of water, which serves as the stationary phase. It is important to note that materials prepared for adsorption chromatogra-

Compounds Class Polarity Scale[1]

Fluorocarbons
Saturated hydrocarbons
Olefins
Aromatics
Halogenated compounds
Ethers
Nitro compounds
Esters – ketones – aldehydes
Alcohols – amines
Amides
Carboxylic acids

From (6), used with permission.
[1]Listed in order of increasing polarity.

31-3
table Eluotropic Series for Alumina

Solvent

1-Pentane
Isooctane
Cyclohexane
Carbon tetrachloride
Xylene
Toluene
Benzene
Ethyl ether
Chloroform
Methylene chloride
Tetrahydrofuran
Acetone
Ethyl acetate
Aniline
Acetonitrile
2-Propanol
Ethanol
Methanol
Acetic acid

From (6), used with permission.

phy must be activated by drying them to remove surface water. Conversely, some of these materials, such as silica gel, may be used for partition chromatography if they are deactivated by impregnation with water or the desired stationary phase. (The terms silica gel or silicic acid actually refer to hydrated silica precipitates, the properties of which can vary widely, depending on the method of preparation used. Depending on the amount of water held, silicic acid may act as an adsorbent or a partition support; usually, both properties contribute in varying degrees to the separations achieved.) One disadvantage of liquid–liquid chromatographic systems is that the liquid stationary phase is often stripped off. This problem can be overcome by

chemically bonding the stationary phase to the support material, as described in the next section.

31.3.3.2.3 Bonded Supports The liquid stationary phase may be covalently attached to a support by a chemical reaction. These bonded phases have become very popular for HPLC use, and a wide variety of both polar and nonpolar stationary phases is now available. (Reactive silanols on the surface of silica gel may be derivatized with alkylamine, alkylnitrile, phenyl, or alkyl groups, as described in Chapter 32.) Again, chromatography performed with a mobile phase less polar than the bonded stationary phase is referred to as normal-phase chromatography. The use of a nonpolar bonded stationary phase (e.g., silica covered with C_8 or C_{18} groups) with a polar solvent (e.g., water–acetonitrile) is called reversed-phase chromatography. The latter has actually become the more widely used of the two methods. (Mechanisms other than partition may be involved in the separation.) Bonded-phase HPLC columns have greatly facilitated the analysis of vitamins in foods and feeds, as discussed in Chapter 3 of reference (7).

31.3.3.3 Ion-Exchange Chromatography

Ion exchange is a separation/purification process occurring naturally, for example, in soils and is utilized in water softeners and deionizers. Three types of separation may be achieved: (1) ionic from nonionic, (2) cationic from anionic, and (3) mixtures of similarly charged species. In the first two cases, one substance binds to the ion-exchange medium, whereas the other substance does not. Batch methods can be used for these two separations; however, chromatography is needed for the third category.

Ion-exchange chromatography may be viewed as a type of adsorption chromatography in which interactions between solute and stationary phase are primarily electrostatic in nature. The stationary phase (ion exchanger) contains fixed functional groups that are either negatively or positively charged (Fig. 31-3a). Exchangeable counterions preserve charge neutrality. A sample ion (or charged sites on large molecules) can exchange with the counterion to become the partner of the fixed charge. Ionic equilibrium is established as depicted in Fig. 31-3b. The functional group of the stationary phase determines whether cations or anions are exchanged. Cation exchangers contain covalently bound negatively charged functional groups, whereas anion exchangers contain bound positively charged groups. The chemical nature of these acidic or basic residues determines how stationary-phase ionization is affected by the mobile-phase pH.

The strongly acidic sulfonic acid moieties (RSO_3^-) of "strong"-cation exchangers are completely ionized

(a)

$$M^+ + (Na^+ \ ^-O_3S—\text{Resin}) \rightleftharpoons (M^+ \ ^-O_3S—\text{Resin}) + Na^+$$

$M^+ = $ Cation

(b)

$$X^- + (Cl^- \ ^+R_4N—\text{Resin}) \rightleftharpoons (X^- \ ^+R_4N—\text{Resin}) + Cl^-$$
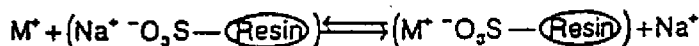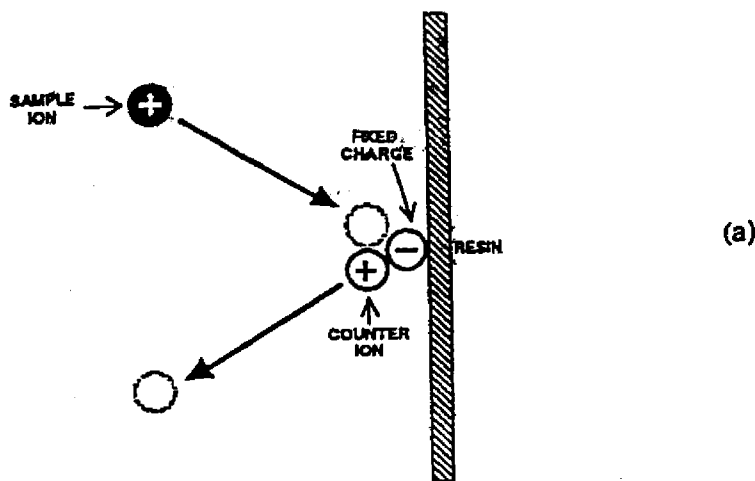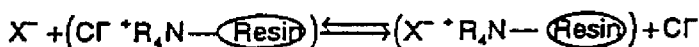
$X^- = $ Anion

31-3 figure

The basis of ion-exchange chromatography. (a) Schematic diagram of the ion-exchange process; (b) ionic equilbria for cation- and anion-exchange processes. [From (6), used with permission.]

at all pH values above 2. Strongly basic quaternary amine groups (RNR'$_3^+$) on "strong"-anion exchangers are ionized at all pH values below 10. Since maximum negative or positive charge is maintained over a broad pH range, the exchange or binding capacity of these stationary phases is essentially constant, regardless of mobile-phase pH. "Weak"-cation exchangers contain weakly acidic carboxylic acid functional groups, (RCO$_2^-$); consequently, their exchange capacity varies considerably between ca. pH 4–10. Weakly basic anion exchangers possess primary, secondary, or tertiary amine residues (R-NHR'$_2^+$), which are deprotonated in moderately basic solution, thereby losing their positive charge and the ability to bind anions. Thus, one way of eluting solutes bound to an ion-exchange medium is to change the mobile-phase pH. A second way to elute bound solutes is to increase the ionic strength (e.g., use NaCl) of the mobile phase, to weaken the electrostatic interactions.

Chromatographic separations by ion exchange are based upon differences in affinity of the exchangers for the ions (or charged species) to be separated. The factors that govern selectivity of an exchanger for a particular ion include the ionic valence, radius, and concentration; the nature of the exchanger (including its displaceable

counterion); and the composition and pH of the mobile phase. To be useful as an ion exchanger, a material must be both ionic in nature and highly permeable. Synthetic ion exchangers are thus crosslinked polyelectrolytes, and they may be inorganic (e.g., aluminosilicates) or, more commonly, organic compounds. Polystyrene, made by crosslinking styrene with divinyl benzene (DVB), may be modified to produce either anion- or cation-exchange resins (Fig. 31-4). Polymeric resins such as these are commercially available in a wide range of particle sizes and with different degrees of crosslinking (expressed as weight percent of DVB in the mixture). The extent of crosslinking controls the rigidity and porosity of the resin, which, in turn, determines its optimal use. Lightly crosslinked resins permit rapid equilibration of solute, but particles swell in water, thereby decreasing charge density and selectivity (relative affinity) of the resin for different ions. More highly crosslinked resins exhibit less swelling, higher exchange capacity, and selectivity, but longer equilibration times. The small pore size, high charge density, and inherent hydrophobicity of the older ion-exchange resins has limited their use to small molecules (MW < 500).

Ion exchangers based on polysaccharides, such as
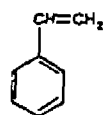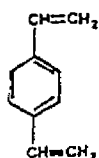
Styrene   Divinylbenzene
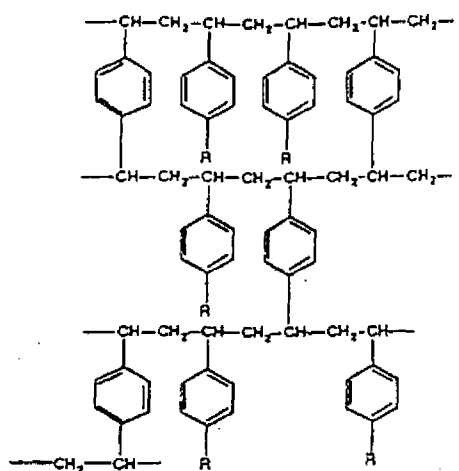
Crosslinked styrene-divinylbenzene copolymer

R=H, Plain polystyrene

R=$CH_2N^+(CH_3)_3Cl^-$, Anion-exchanger

R=$SO_3^-H^+$, Cation-exchanger

31-4 figure Chemical structure of polystyrene-based ion-exchange resins.

cellulose, dextran, or agarose, have proven very useful for the separation and purification of large molecules, such as proteins and nucleic acids. These materials, called gels because they are much softer than polystyrene resins, may be derivatized with strong or with weakly acidic or basic groups via OH moieties on the polysaccharide backbone (Fig. 31-5). They have much larger pore sizes and lower charge densities than the older synthetic resins.

Food-related applications of ion-exchange chromatography include the separation of amino acids, sugars, alkaloids, and proteins. Fractionation of amino acids in protein hydrolyzates was initially carried out by ion-exchange chromatography; automation of this process led to the development of commercially produced amino acid analyzers (see Chapter 16). Sugars,

complexed with borate to form negatively charged species, can be separated on columns of strong-anion-exchange resin in the borate form. Many drugs, fatty acids, and the acids of fruit, being ionizable compounds, may be chromatographed in the ion-exchange mode.

31.3.3.4 Size-Exclusion Chromatography

Size-exclusion chromatography (SEC), also known as molecular exclusion, gel permeation (GPC), and gel-filtration chromatography (GFC), is probably the easiest mode of chromatography to perform and to understand. It is widely used in the biological sciences for the resolution of macromolecules, such as proteins and carbohydrates, and also is used for the fractionation and characterization of synthetic polymers. Unfortunately, nomenclature associated with this separation mode developed independently in the literature of the life sciences and in the field of polymer chemistry, resulting in inconsistencies.

In the ideal SEC system, molecules are separated solely on the basis of their size; no interaction occurs between solutes and the stationary phase. (In the event that solute–support interactions do occur, the separation mode is termed nonideal SEC.) The stationary phase in SEC consists of a column packing material that contains pores comparable in size to the molecules to be fractionated. Solutes too large to enter the pores travel with the mobile phase in the interstitial space (between particles) outside the pores. Thus, the largest molecules are eluted first from an SEC column. The volume of the mobile phase in the column, termed the column void volume, V_o, can be measured by chromatographing a very large (totally excluded) species, such as Blue Dextran, a dye of MW = 2×10^6.

As solute dimensions decrease, approaching those of the packing pores, molecules begin to diffuse into the packing particles and, consequently, are slowed down. Solutes of low molecular weight (e.g., glycyltyrosine) that have free access to all the available pore volume are eluted in the volume referred to as V_t. This value, V_t, which is equal to the column void volume, V_o, plus the volume of liquid inside the sorbent pores, V_i, is referred to as the total permeation volume of the packed column ($V_t = V_o + V_i$). These relationships are illustrated in Fig. 31-6. Solutes are ideally eluted between the void volume and the total liquid volume of the column. Because this volume is limited, only a relatively small number of solutes (ca. 10) can be completely resolved by SEC under ordinary conditions.

The behavior of a molecule in a size-exclusion column may be characterized in several different ways. Each solute exhibits an elution volume, V_e, as illustrated in Fig. 31-6. However, V_e depends on column dimensions and the way in which the column was

Derivatization sites

(a)

{—OCH₂COO⁻

Carboxymethyl- (CM)
(weak acid)

{—OCH₂CH₂ṄH⟨CH₂CH₃ / CH₂CH₃⟩

Diethylaminoethyl- (DEAE)
(weak base)

{—O—P(=O)(O⁻)—O⁻

Phospho- (P)
(intermediate acid)

(b)

{—OCH₂CH₂⁺NCH₂CH⟨OH / CH₃⟩ (CH₂CH₃)(CH₂CH₃)

Quaternaryaminoethyl- (QAE)
(strong base)

{—OCH₂CH₂SO₃⁻

Sulfoethyl- (SE)
(strong acid)
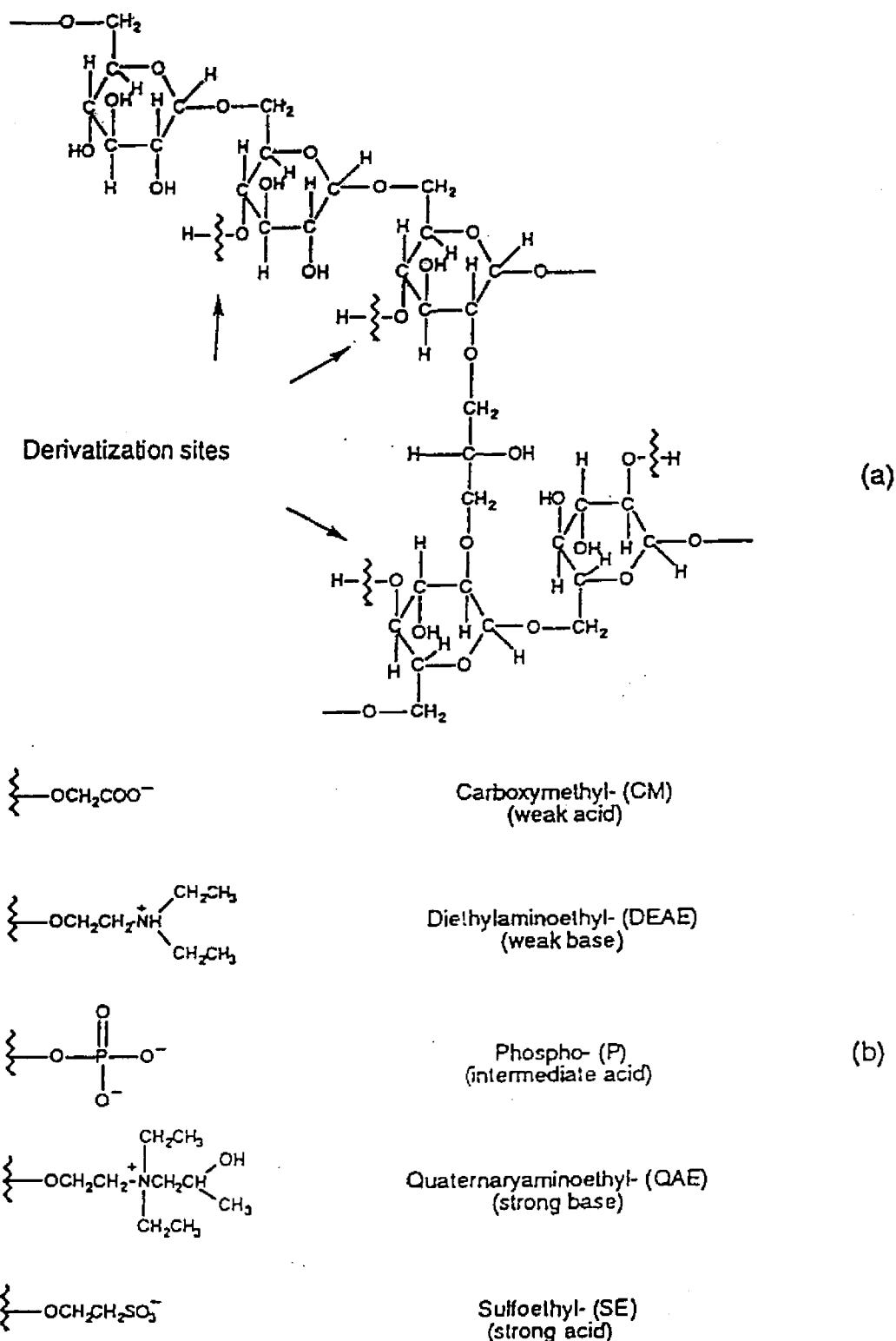
31-5
figure

Chemical structure of one polysaccharide-based ion-exchange resin. (a) Matrix of crosslinked dextran ("Sephadex," Pharmacia Biotech Inc, Piscataway NJ); (b) functional groups that may be used to impart ion-exchange properties to the matrix.

—void volume— —internal pore volume (v_i)—
————————total permeation volume————————
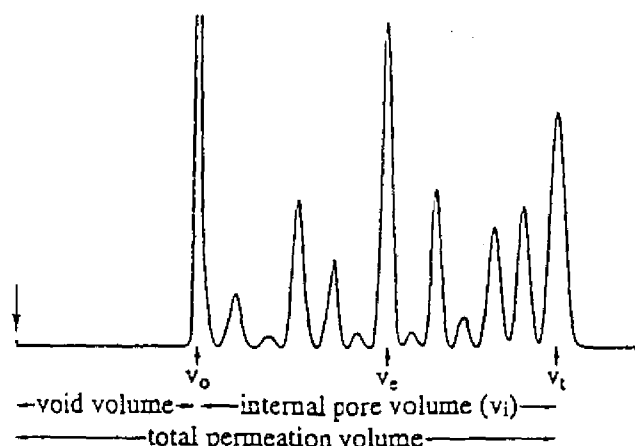
31-6 figure Schematic elution profile illustrating some of the terms used in size-exclusion chromatography. [Adapted from (5), p. A271, with kind permission from Elsevier Science-NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands.]

packed. The available partition coefficient is used to define solute behavior independent of these variables:

$$K_{av} = (V_e - V_o)/(V_t - V_o) \qquad [2]$$

where:

K_{av} = available partition coefficient
V_e = elution volume of solute
V_o = column void volume
V_t = total permeation volume of column

The value of K_{av} calculated from experimental data for a solute chromatographed on a given SEC column defines the proportion of pores that can be occupied by that molecule. For a large, totally excluded species, such as Blue Dextran or DNA, $V_e = V_o$ and $K_{av} = 0$. For a small molecule with complete access to the internal pore volume, such as glycyltyrosine, $V_e = V_t$ and $K_{av} = 1$.

For each size-exclusion packing material, a plot of K_{av} versus the logarithm of the molecular weight for a series of solutes, similar in molecular shape and density, will give an S-shaped curve (Fig. 31-7). (In the case of proteins, K_{av} is actually better related to the Stokes radius, the average radius of the protein in solution.) The central, linear portion of this curve describes the fractionation range of the matrix, wherein maximum separation among solutes of similar molecular weight is achieved. This correlation between solute elution behavior and molecular weight (or size) forms the basis for a widely used method for characterizing large molecules such as proteins and polysaccharides. A size-exclusion column is calibrated with a series of solutes of known molecular weight (or Stokes radius) to obtain a curve similar to that shown in Fig. 31-7. The

value of K_{av} for the unknown is then determined, and an estimate of molecular weight (or size) of the unknown is made by interpolation of the calibration curve.

Column packing materials for size-exclusion chromatography can be divided into two groups: semi-rigid, hydrophobic media, and soft, hydrophilic gels. The former are usually derived from polystyrene and are used with organic mobile phases (GPC or non-aqueous SEC) for the separation of polymers, such as rubbers and plastics. Soft gels, polysaccharide-based packings, are typified by Sephadex, a crosslinked dextran (see Fig. 31-5A). These materials are available in a wide range of pore sizes and are useful for the separation of water-soluble substances in the molecular weight range $1-25 \times 10^7$. In selecting an SEC column packing, both the purpose of the experiment and size of the molecules to be separated must be considered. For example, if the objective is to determine molecular weight, a packing is chosen such that its fractionation range encompasses the anticipated molecular weight of the solute.

As discussed previously, SEC can be used, directly, to fractionate mixtures or, indirectly, to obtain information about a dissolved species. In addition to molecular weight estimations, SEC is used to determine the molecular weight distribution of natural and synthetic polymers, such as dextrans and gelatin preparations. Fractionation of biopolymer mixtures is probably the most widespread use of SEC, since the mild elution conditions employed rarely cause denaturation or loss of biological activity. It is also a fast, efficient alternative to dialysis for desalting solutions of macromolecules, such as proteins.

31.3.3.5 Affinity Chromatography

Affinity chromatography is unique in that separation is based on the specific, reversible interaction between a solute molecule and a ligand immobilized on the chromatographic stationary phase. While discussed here as a separate type of chromatography, affinity chromatography could be viewed as the ultimate extension of adsorption chromatography. Although the basic concepts of so-called biospecific adsorption were known as early as 1910, they were not perceived as potentially useful laboratory tools until ca. 1968.

Affinity chromatography usually involves immobilized biological materials as the stationary phase. These ligands can be antibodies, enzyme inhibitors, lectins, or other molecules that selectively and reversibly bind to complementary analyte molecules in the sample. Separation exploits the lock and key binding of biological systems. Although both ligands and the species to be isolated are usually biological macromolecules, the term affinity chromatography also encompasses other systems, such as separation of small
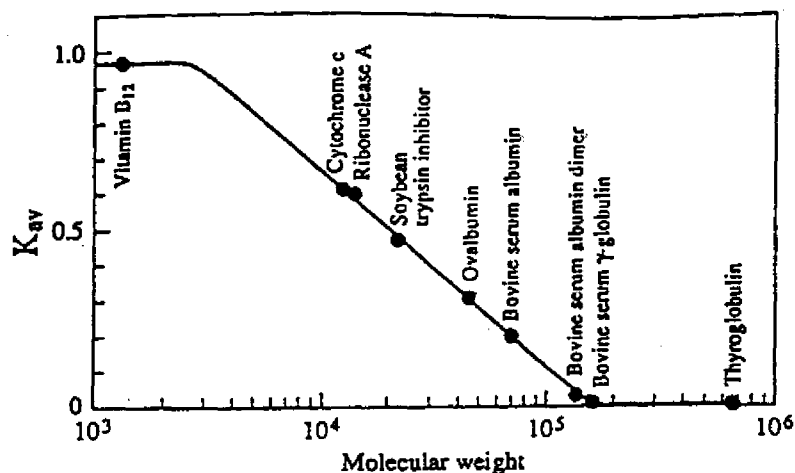
31-7 figure   Relationship between K_{av} and log (molecular weight) for globular proteins chromatographed on a column of Sephadex G-150 Superfine. (Reproduced by permission of Pharmacia Biotech, Inc., Piscataway, NJ.)

molecules containing cis-diol groups via phenylboronic acid moieties on the stationary phase.

The principles of affinity chromatography are illustrated in Fig. 31-8. A ligand chosen because of the specificity and strength of interaction between itself and the molecule to be isolated (analyte) is immobilized on a suitable support material. As the sample is passed through this column, molecules that are complementary to the bound ligand are adsorbed while other sample components are eluted. Bound analyte is subsequently eluted via a change in the mobile-phase composition. (For example, changing the pH of the mobile phase often dissociates an enzyme-inhibitor complex.) After reequilibration with the initial mobile phase, the stationary phase is ready to be used again. The ideal support for affinity chromatography should be a porous, stable, high-surface-area material that does not adsorb anything itself. Thus, polymers such as agarose, cellulose, dextran, and polyacrylamide are used, as well as controlled-pore glass.

Affinity ligands are usually attached to the support or matrix by covalent bond formation, and optimum reaction conditions often must be found empirically. Immobilization generally consists of two steps: activation and coupling. During the activation step, a reagent reacts with functional groups on the support, such as hydroxyl moieties, to produce an activated matrix. After removal of excess reagent, the ligand is coupled to the activated matrix. (Preactivated supports are commercially available, and their availability has greatly increased the use of affinity chromatography). The coupling reaction most often involves free amino groups on the ligand, although other functional groups can be used. When small molecules such as phenylboronic acid are immobilized, a spacer arm (containing at least four to six methylene groups) is used to hold

the ligand away from the support surface, enabling it to reach into the binding site of the analyte.

Ligands for affinity chromatography may be either specific or general (i.e., group specific). Specific ligands, such as antibodies, bind only one particular solute. General ligands, such as nucleotide analogs and lectins, bind to certain classes of solutes. For example, the lectin concanavalin A binds to all molecules that contain terminal glucosyl and mannosyl residues. Bound solutes then can be separated as a group or individually, depending upon the elution technique used. Some of the more common general ligands are listed in Table 31-4. Although less selective, general ligands provide greater convenience.

Elution methods for affinity chromatography may be divided into nonspecific and (bio)specific methods. Nonspecific elution involves disrupting ligand analyte binding by changing the mobile-phase pH, ionic strength, dielectric constant, or temperature. If additional selectivity in elution is desired, for example in the case of immobilized general ligands, a biospecific elution technique is used. Free ligand, either identical to or different from the matrix-bound ligand, is added to the mobile phase. This free ligand competes for binding sites on the analyte. For example, glycoproteins bound to a concanavalin A (lectin) column can be eluted by using buffer containing an excess of lectin. In general, the eluent ligand should display greater affinity for the analyte of interest than the immobilized ligand.

In addition to protein purification, affinity chromatography may be used to separate supramolecular structures such as cells, organelles, and viruses; concentrate dilute protein solutions; investigate binding mechanisms; and determine equilibrium constants. Affinity chromatography has been useful especially in

A SPACER

ANALYTE

LIGAND

SUPPORT

B

C

D
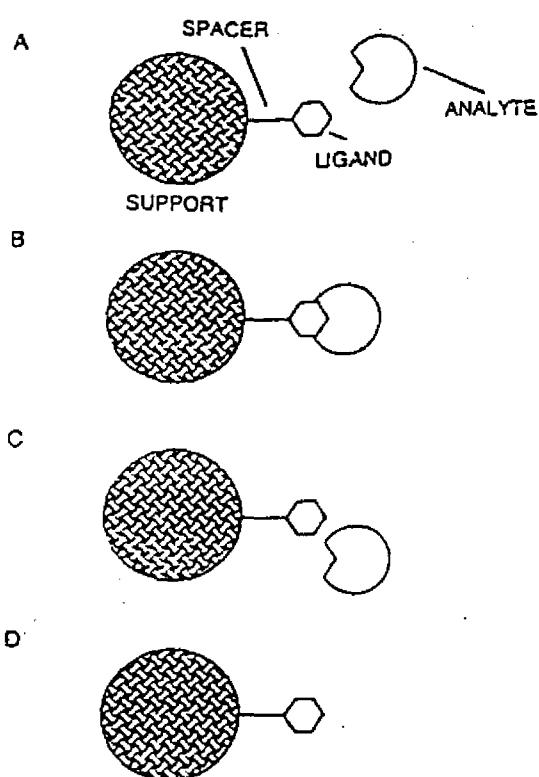
31-8
figure

Principles of bioselective affinity chromatography. (A) The support presents the immobilized ligand to the analyte to be isolated. (B) The analyte makes contact with the ligand and attaches itself. (C) The analyte is recovered by the introduction of an eluent, which dissociates the complex holding the analyte to the ligand. (D) The support is regenerated, ready for the next isolation. [Reprinted from (5), p. A311, with kind permission from Elsevier Science-NL, Sara Burgerhartsraat 25, 1055 KV Amsterdam, The Netherlands.]

the separation and purification of enzymes and glycoproteins. In the case of the latter, carbohydrate-derivatized adsorbents are used to isolate specific lectins, such as concanavalin A, and lentil or wheat-germ lectin. The lectin then may be coupled to agarose, such as concanavalin A– or lentil lectin–agarose, to provide a stationary phase for the purification of specific glycoproteins, glycolipids, or polysaccharides.

31.3.4 Chromatographic Techniques

The same general principles of chromatography apply, regardless of the specific method or technique used. Paper, thin-layer, and column liquid chromatography all utilize a liquid mobile phase, but the physical form of the stationary phase is quite different in each case. Supercritical fluid chromatography is an analytical technique similar to liquid chromatography, except that a supercritical fluid, instead of a liquid, is used as the mobile phase.

31.3.4.1 Paper Chromatography

Paper chromatography was introduced in 1944. Although adsorption by the paper itself has been utilized, paper generally serves only as a support for the liquid stationary phase (partition chromatography). To carry out this technique, the dissolved sample is applied as a small spot or streak one-half inch or more from the edge of a strip or square of filter paper (usually cellulose) and is allowed to dry. The strip is then suspended in a closed container, the atmosphere of which is saturated with the developing solvent (mobile phase), and the paper chromatogram is developed. The end closer to the sample is placed in contact with solvent, which then travels up or down the paper by capillary action

31-4
table General Affinity Ligands and Their Specificities

Ligand	Specificity
Cibacron Blue F3G-A dye, derivatives of AMP, NADH, and NADPH	Certain dehydrogenases via binding at the nucleotide binding site
Concanavalin A, lentil lectin, wheat germ lectin	Polysaccharides, glycoproteins, glycolipids, and membrane proteins containing sugar residues of certain configurations
Soybean trypsin inhibitor, methyl esters of various amino acids, ɔ-amino acids	Various proteases
Phenylboronic acid	Glycosylated hemoglobins, sugars, nucleic acids, and other cis-diol-containing substances
Protein A	Many immunoglobin classes and subclasses via binding to the F_c region
DNA, RNA, nucleosides, nucleotides	Nucleases, polymerases, nucleic acids

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(depending on whether ascending or descending development is used), separating sample components in the process. When the solvent front has traveled the length of the paper, the strip is removed from the developing chamber and the separated zones are detected by an appropriate method.

The stationary phase in paper partition chromatography is usually water. However, the support may be impregnated with a nonpolar organic solvent and developed with polar solvents or water (reversed-phase paper chromatography). In the case of complex sample mixtures, a two-dimensional technique may be used. The sample is spotted in one corner of a square sheet of paper, and one solvent is used to develop the paper in one direction. The chromatogram is then dried, turned 90°, and developed again, using a second solvent of different polarity. Another means of improving resolution is the use of ion-exchange papers. Both paper that has been impregnated with ion-exchange resin and paper in which cellulose hydroxyl groups have been derivatized (with acidic or basic moieties) are available commercially.

In paper and thin-layer (planar) chromatography, components of a mixture are characterized by their R_f value, where:

$$R_f = \frac{\text{Distance moved by component}}{\text{Distance moved by solvent}} \quad [3]$$

Unfortunately, R_f values are not always constant for a given solute/sorbent/solvent, but depend on many factors, such as the quality of the stationary phase, layer thickness, humidity, development distance, and temperature.

31.3.4.2 Thin-Layer Chromatography

Thin-layer chromatography (TLC), first described in 1938, has largely replaced paper chromatography because it is faster, more sensitive, and more reproducible. (Both of these techniques may be referred to as planar chromatography.) The resolution in TLC is greater than in paper chromatography because the particles on the plate are smaller and more regular than paper fibers. Experimental conditions can be easily varied to achieve separation and can be scaled up for use in column chromatography. (Thin-layer and column procedures are not necessarily interchangeable, due to differences such as the use of binders with TLC plates, vapor-phase equilibria in a TLC tank, etc.) Some distinct advantages of TLC include high sample throughput and low cost; the possibility to analyze several samples and standards simultaneously; and minimal sample preparation (since the stationary phase is disposable). In addition, a plate may be stored for later identification and quantitation.

Thin-layer chromatography has been applied to

the analysis of lipids (see Chapter 14). High performance liquid chromatography of lipids is complicated by the lack of chromophores that permit UV-Vis detection, and most GC analyses require prior derivatization; however, many good lipid detection reagents are available for TLC. Thin-layer chromatography is applied in many fields, including environmental, clinical, forensic, pharmaceutical, food, flavors, and cosmetics. Within the food industry, TLC may be used for quality control. For example, corn and peanuts are tested for aflatoxins/mycotoxins prior to their processing into corn meal and peanut butter, respectively. Recent applications of TLC to the analysis of a variety of compounds, including lipids, toxins, pesticides, carbohydrates, vitamins, amino acids, peptides and proteins, are reviewed by Sherma (9).

31.3.4.2.1 General Procedures Thin-layer chromatography utilizes a thin (ca. 250 µm thick) layer of sorbent or stationary phase bound to an inert support in a planar configuration. The support is often a glass plate (traditionally, 20 cm × 20 cm) but plastic sheets and aluminum foil also are used. Precoated plates, of different layer thicknesses, are commercially available in a wide variety of sorbents, including chemically modified silicas. (Plates are seldom hand-coated today.) Four frequently used TLC sorbents are silica gel, alumina, diatomaceous earth, and cellulose. Many separations achieved by paper chromatography can be transferred to TLC on cellulose. Modified silicas for TLC may contain polar or nonpolar groups, analogous to bonded phases for column chromatography (see section 31.3.3.2.3.), and both normal and reversed-phase thin-layer separations may be carried out. High performance thin-layer chromatography (HPTLC) simply refers to TLC performed using plates coated with smaller, more uniform particles. This permits better separations in shorter times.

If adsorption TLC is to be performed, the sorbent is first activated by drying for a specified time and temperature. Sample (in carrier solvent) is applied as a spot or streak 1–2 cm from one end of the plate. After evaporation of carrier solvent, the TLC plate is placed in a closed developing chamber with the end of the plate nearest the spot in the solvent at the bottom of the chamber. Traditionally, solvent migrates up the plate (ascending development) by capillary action and sample components are separated. After the TLC plate has been removed form the chamber and solvent allowed to evaporate, the separated bands are made visible (visualized) or detected by other means. Specific chemical reactions (derivatization), which may be carried out either before or after chromatography, often are used for this purpose. Two examples are reaction with sulfuric acid to produce a dark charred area (a destructive chemical method), and the use of iodine

vapor to form a colored complex (a nondestructive method inasmuch as the colored complex is usually not permanent). Common physical detection methods include the measurement of absorbed or emitted electromagnetic radiation, e.g., fluorescence, and measurement of β-radiation from radioactively labeled compounds. Biological methods or biochemical inhibition tests can be used to detect toxicologically active substances. An example is measuring the inhibition of cholinesterase activity by organophosphate pesticides.

Quantitative evaluation of thin-layer chromatograms may be performed (1) in situ (directly on the layer) by using a densitometer or (2) after scraping a zone off the plate, eluting compound from the sorbent, and analyzing the resultant solution, e.g., by liquid scintillation counting.

31.3.4.2.2 Factors Affecting Thin-Layer Separations

In both planar and column liquid chromatography, the nature of the compounds to be separated determines what type of stationary phase is used. Separation can occur by adsorption, partition, ion-exchange, size-exclusion, or multiple mechanisms as previously discussed in section 31.3.3. Table 31-5 lists the separation mechanisms involved in some typical applications on common TLC sorbents.

Although selection of both mobile and stationary phases determine the success of a given TLC separation, the rationale behind choice of mobile phase for a particular fractionation often is not described. Solvents for TLC separations should be selected on the basis of their chemical characteristics and solvent strength (a measure of interaction between solvent and sorbent; see section 31.3.3.1). In simple adsorption TLC, the higher the solvent strength, the greater the R_f value of the solute. One usually tries to use a mobile phase such that R_f values of 0.3–0.7 are obtained. (Although single solvent mobile phases may provide adequate mobility, they often do not give adequate separation.) Fortu-

nately for the beginner, mobile phases have been developed for the separation of various compound classes on specific sorbents; see, for example, Table 7.1 in reference (12).

In addition to the sorbent and solvent, several other factors must be considered when performing thin-layer (or paper) chromotography. These include the type of developing chamber used, vapor phase conditions (saturated versus unsaturated), development mode (ascending, descending, horizontal, radial, etc.), and development distance.

31.3.4.3 Column Liquid Chromatography

Column chromatography is the most useful method of separating compounds in a mixture. Fractionation of solutes occurs as a result of differential migration through a closed tube of stationary phase, and analytes can be monitored while the separation is in progress. This section of the chapter will cover general procedures, theory, and the quantitation of data from column liquid chromatography.

31.3.4.3.1 General Procedures

A system for low pressure (i.e., performed at or near atmospheric pressure) column liquid chromatography is illustrated in Fig. 31-9. (While the procedure outlined below is applicable to column chromatography in general, the reader is referred to subsequent chapters for details specific to HPLC or GC).

Having selected a stationary and mobile phase suitable for the separation problem at hand, the analyst must first prepare the stationary phase (resin, gel, or packing material) for use according to the supplier's instructions. (For example, the stationary phase often must be hydrated or preswelled in the mobile phase.) The prepared stationary phase then is packed into a column (usually glass), the length and diameter of which are determined by the amount of sample to be

31-5 table

Thin-Layer Chromatography Sorbents and Mode of Separation

Sorbent	Chromatographic Mechanism	Typical Application
Silica gel	Adsorption	Steroids, amino acids, alchchols, hydrocarbons, lipids, aflatoxins, oile acics, vitamins, alkaloids
Silica gel RP	Reversed phase	Fatty acids, vitamins, steroids, hormones, carotenoids
Cellulose, kieselguhr	Partition	Carbohydrates, sugars, alcchcls, amino acids, carboxylic acids, fatty acics
Aluminum oxide	Adsorption	Amines, alcohols, steroids, lipics, aflatoxins, bile acids, vitamins, a.ka c ds
PEI cellulose[1]	Ion exchange	Nucleic acids, nucleotides, nucleosides, purines, pyrimidines
Magnesium silicate	Adsorption	Steroids, pesticides, lipids, alkaloids

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[1]PEI cellulose refers to cellulose derivatized with polyethyleneimine (PEI).
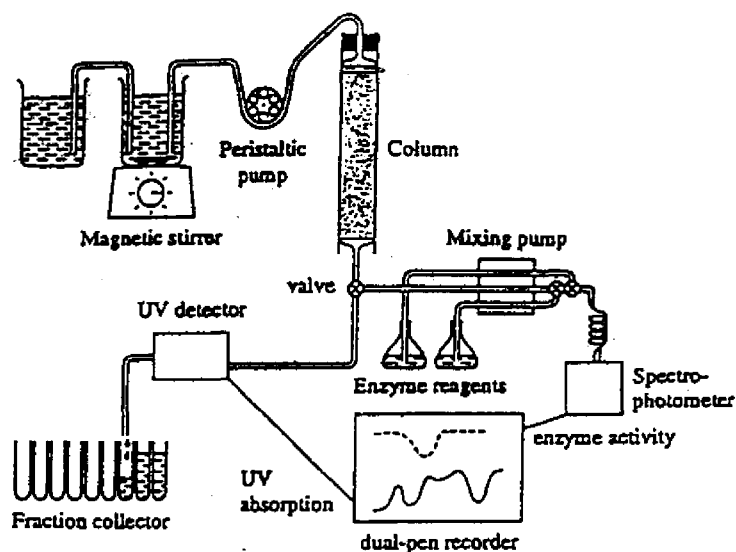
31-9 figure A system for low pressure column liquid chromatography. In this diagram, the column effluent is being split between two detectors in order to monitor both enzyme activity (at right) and UV absorption (at left). The two tracings can be recorded simultaneously by using a dual-pen recorder. [Adapted from (8), with permission.]

loaded, the separation mode to be used, and the degree of resolution required. Adsorption columns may be either dry- or wet-packed; other types of columns are wet-packed. The most common technique for wet-packing involves making a slurry of the adsorbent with the solvent and pouring this into the column. As the sorbent settles, excess solvent is drained off and additional slurry is added. This process is repeated until the desired bed height is obtained. (There is a certain art to pouring uniform columns and no attempt is made to give details here.) If the packing solvent is different from the initial eluting solvent, the column must be thoroughly washed (equilibrated) with the starting mobile phase.

The sample to be fractionated, dissolved in a minimum volume of mobile phase, is applied in a layer at the top (or head) of the column. Classical or low pressure chromatography utilizes only gravity flow or a peristaltic pump to maintain a flow of mobile phase (eluent or eluting solvent) through the column. In the case of a gravity-fed system, eluent is simply siphoned from a reservoir into the column. The flow rate is governed by the hydrostatic pressure, measured as the distance between the level of liquid in the reservoir and the level of the column outlet. If eluent is fed to the column by a peristaltic pump (see Fig. 31-9), the flow rate is determined by the pump speed and, thus, regulation of hydrostatic pressure is not necessary.

The process of passing the mobile phase through the column is called elution, and the portion that emerges from the outlet end of the column is sometimes called the eluate (or effluent). Elution may be iso-

cratic (constant mobile-phase composition) or a gradient may be used. Gradient elution refers to changing the mobile phase (e.g., increasing solvent strength or pH) during elution in order to enhance resolution and decrease analysis time. The change may be continuous or stepwise. Gradients of increasing ionic strength are extremely valuable in ion-exchange chromatography. Gradient elution is commonly used for desorbing large molecules, such as proteins, which can undergo multiple-site interaction with a stationary phase. As elution proceeds, components of the sample are selectively retarded by the stationary phase according to one (or more) of the mechanisms discussed earlier, and thus are eluted at different times.

The column effluent may be directed through a detector and then into tubes, changed at intervals by a fraction collector. The detector response, in the form of an electrical signal, may be recorded (the chromatogram) and used for qualitative or quantitative analysis, as discussed in more detail later. The fraction collector may be set to collect eluate at specified time intervals or after a certain volume or number of drops have been collected. Components of the sample that have been chromatographically separated in this manner then can be analyzed as needed.

31.3.4.3.2 Qualitative Analysis The volume of liquid required to elute a compound from a liquid chromatography column is called the retention volume, V_R. The associated time is the retention time, t_R. Comparing V_R or t_R to that of standards chromatographed under identical conditions often enables one to identify

an unknown compound. (One should remember that different compounds may have identical retention times.) A related technique is to spike the unknown sample with a known compound and compare chromatograms of the original and spiked samples to see which peak has increased. In most cases, it will be necessary to collect the peak(s) of interest and establish their identity by another analytical method.

Often it is necessary to compare chromatograms obtained from two different systems or columns. Differences in column dimensions, loading, temperature, flow rate, system dead-volume, and detector geometry may lead to discrepancies for uncorrected retention data. By subtracting the time required for the mobile phase or a nonretained solute (t_m or t_o) to travel through the column to the detector, one obtains an adjusted retention time, t'_R (or volume) as depicted in Fig. 31-10. The adjusted retention time (or volume) corrects for differences in system dead-volume; it may be thought of as the time the sample spends adsorbed on the stationary phase.

A simple, reliable method for the identification of peaks is to use relative retention as expressed by the separation factor, α. Values for α (Fig. 31-10) depend only on temperature and the stationary phase and mobile phase used.

31.3.4.3.3 Separation and Resolution

1. Overview. The goal of chromatography is to segregate components of a sample into separate bands or peaks as they migrate through the column. The resolution of two peaks from each other is defined as

$$R_s = \frac{2\Delta t}{w_2 + w_1} \qquad [4]$$

where:

R_s = resolution
Δt = difference between retention times of peaks 1 and 2
w_2 = width of peak 2 at baseline
w_1 = width of peak 1 at baseline

Figure 31-11 illustrates the measurement of peak width (part A) and the values necessary for calculating resolution (part B). (Retention and peak or band width must be expressed in the same units, i.e., time or volume.)

Chromatographic resolution is a function of column efficiency, selectivity, and the capacity factor. Mathematically, this relationship is expressed as:

$$R_s = \underbrace{1/4\sqrt{N}}_{a}\underbrace{\left(\frac{\alpha - 1}{\alpha}\right)}_{b}\underbrace{\left(\frac{k'}{k' + 1}\right)}_{c} \qquad [5]$$

where:

a = the column efficiency term
b = the column selectivity term
c = the capacity term

These terms, and factors that contribute to them, will be discussed in the following paragraphs.

2. Efficiency. If faced with the problem of improving resolution, a chromatographer should first examine the efficiency of the column. An efficient column keeps the bands from spreading and/or gives narrow peaks. Column efficiency can be quantitated by

Measurement of chromatographic retention. [Adapted from (6), with permission.]

(a)

(b)
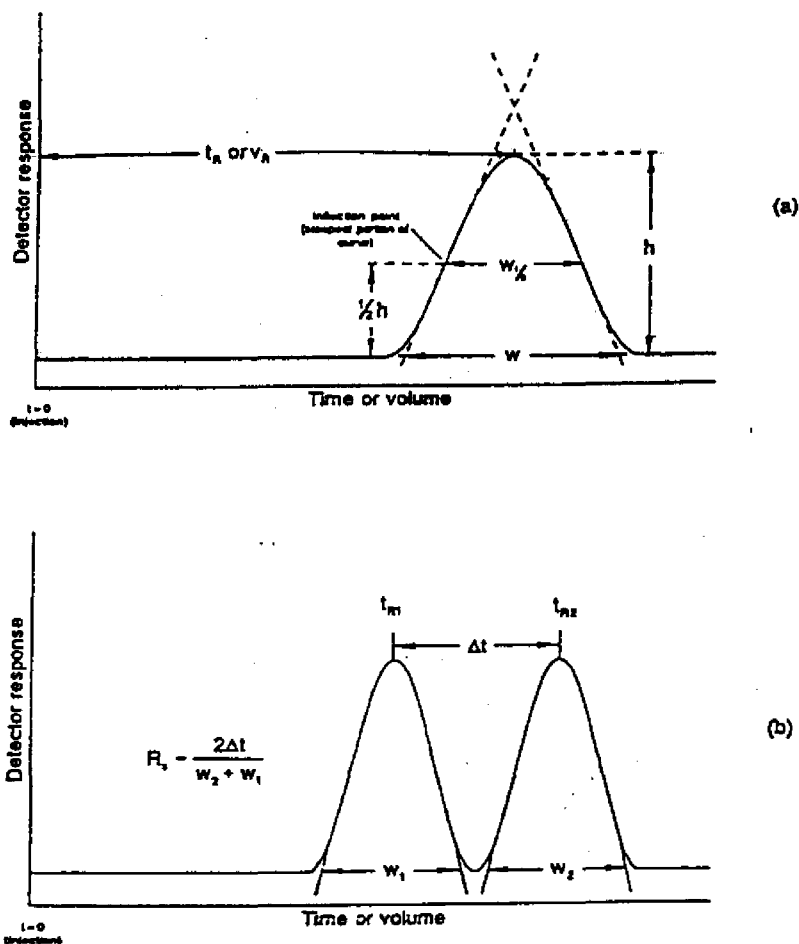
31-11
figure

Measurement of peak width and its contribution to resolution. (a) Idealized Gaussian chromatogram, illustrating the measurement of w and $w_{1/2}$. (b) the resolution of two bands is a function of both their relative retentions and peak widths. [Adapted from (6), with permission.]

$$N = \left(\frac{t_R}{\sigma}\right)^2 = 16\left(\frac{t_R}{w}\right)^2 = 5.5\left(\frac{t_R}{w_{1/2}}\right)^2 \qquad [6]$$

where:

N = number of theoretical plates

t_R = retention time

σ = standard deviation for a Gaussian peak

w = peak width at baseline ($w = 4\sigma$)

$w_{1/2}$ = peak width at half height

The measurement of t_R, w, and $w_{1/2}$ is illustrated in Fig. 31-11. (Retention volume may be used instead of t_R; in this case, band width is also measured in units of volume.) Although few peaks are actually Gaussian in shape, normal practice is to treat them as if they were. In the case of peaks that are incompletely resolved or slightly asymmetric, peak width at half-height is more accurate than peak width at baseline.

The value N calculated from the above equation is called the number of *theoretical plates*. The theoretical

plate concept, borrowed from distillation theory, can best be understood by viewing chromatography as a series of equilibrations between mobile and stationary phases, analogous to countercurrent distribution. A column, then, would consist of N segments (theoretical plates) with one equilibration occurring in each. As a first approximation, N is independent of retention time and is therefore a useful measure of column performance. One method of monitoring column performance over time is to chromatograph a standard compound periodically, under constant conditions, and to compare the values of N obtained. Band broadening due to column deterioration will result in a decrease of N.

The number of theoretical plates is generally proportional to column length. Since columns are available in various lengths, it is useful to have a measure of column efficiency that is independent of column length. This may be expressed as:

$$HETP = \frac{L}{N} \qquad [7]$$

where:

HETP = height equivalent to a theoretical plate
L = column length
N = number of theoretical plates

The so-called height equivalent to a theoretical plate (HETP) is sometimes more simply described as plate height (H). If a column consisted of discrete segments, HETP would be the height of each imaginary segment. Small plate height values (a large number of plates) indicate good efficiency of separation.

Obviously, plate theory is an oversimplification because chromatography is a continuous process. Columns are not divided into discrete segments and equilibration is not infinitely fast. Also, columns often behave as if they have a different number of plates for different solutes in a mixture. A more realistic description of the movement of solutes through a chromatography column takes into account the finite rate at which a solute can equilibrate itself between stationary and mobile phases. Thus, band shape depends on the rate of elution and is affected by solute diffusion. Any mechanism that causes a band of solute to broaden will increase HETP, consequently decreasing column efficiency. The various factors that contribute to plate height are expressed by the Van Deemter equation:

$$HETP = Au^{1/3} + \frac{B}{u} + Cu \qquad [8]$$

where:

HETP = height equivalent to a theoretical plate
A, B, C = constants
u = mobile phase velocity

The constants A, B, and C are characteristic for a given column, mobile phase, and temperature. The A term results from eddy diffusion or multiple flowpaths. Eddy diffusion refers to the different microscopic flowstreams that the mobile phase can take between particles in the column (analogous to eddy streams around rocks in a brook). Sample molecules can thus take different paths as well, depending on which flowstreams they follow. As a result, solute molecules spread from an initially narrow band to a broader area within the column. Eddy diffusion may be minimized by good column packing techniques and the use of small diameter particles of narrow particle size distribution. The B term of the Van Deemter equation (sometimes called the longitudinal diffusion term) exists because all solutes diffuse from an area of high concentration to one of low concentration. In liquid chromatography, the contribution of this term to HETP is small except at low flow velocities. (The more time a solute spends on the column, the greater will be its diffusive spreading.) The C (mass transfer) term arises from the finite time

required for solute to equilibrate between the mobile and stationary phases. If the stationary phase consists of porous particles (see Chapter 32, Fig. 32-3), a sample molecule entering a pore ceases to be transported by the solvent flow and moves by diffusion only. Subsequently, this solute molecule may diffuse back to the mobile phase flow or it may interact with the stationary phase. In either case, solute molecules inside the pores are slowed down relative to those outside the pores and band broadening occurs. Contributions to HETP from the C term can be minimized by using porous particles of small diameter or pellicular packing materials (Chapter 32, section 32.2.3.2.2). As expressed by the Van Deemter equation, mobile phase velocity, u, contributes to plate height in opposing ways—increasing the flow rate increases eddy diffusion and the mass transfer component (A and C terms), but decreases longitudinal diffusion (B term). A Van Deemter plot (Chapter 33, Fig. 33-14) may be used to determine the mobile phase flow velocity at which plate height is minimized and column efficiency is maximized.

3. Selectivity. Chromatographic resolution depends on selectivity as well as efficiency. Column selectivity refers to the distance, or relative separation, between two peaks and is given by

$$\alpha = \frac{t_{R2} - t_o}{t_{R1} - t_o} = \frac{t'_{R2}}{t'_{R1}} = \frac{K_2}{K_1} \qquad [9]$$

where:

α = separation factor
t_{R1} and t_{R2} = retention times of components 1 and 2, respectively
t_o (or t_m) = retention time of unretained components (solvent front)
t'_{R1} and t'_{R2} = adjusted retention times of components 1 and 2, respectively
K_1 and K_2 = distribution coefficients of components 1 and 2, respectively

Retention times (or volumes) are measured as shown in Fig. 31-10. The time, t_o, can be measured by chromatographing a solute that is not retained under the separation conditions (i.e., travels with the solvent front). When this parameter is expressed in units of volume, V_o or V_m, it is known as the dead-volume of the system. Selectivity is a function of the stationary and/or mobile phase. For example, selectivity in ion-exchange chromatography is influenced by the nature and number of ionic groups on the matrix but also can be manipulated via pH and ionic strength of the mobile phase. Good selectivity is probably more important to a given separation than high efficiency (Fig. 31-12), since resolution is directly related to selectivity but is quadratically related to efficiency; thus a fourfold increase in N is needed to double R_s (Equation [5]).

504

(a)

(b)

(c)
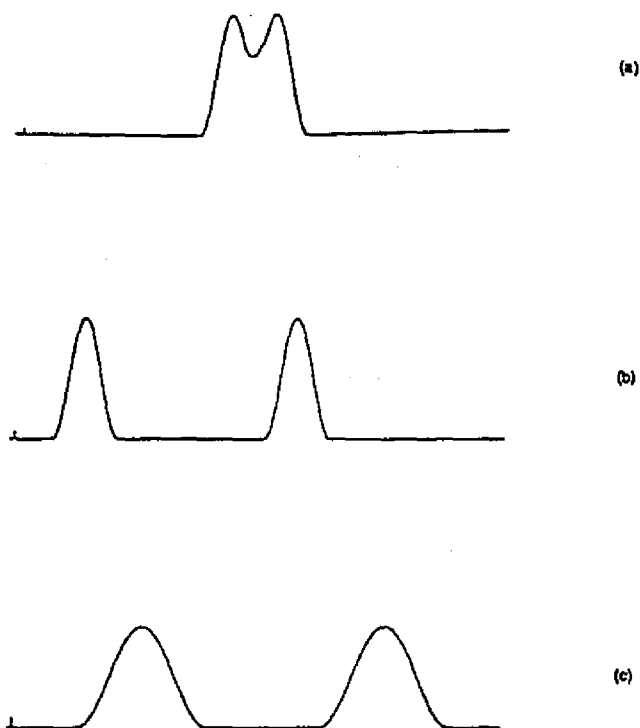
31-12
figure

Chromatographic resolution: efficiency versus selectivity. (a) Poor resolution; (b) good resolution due to high column efficiency; (c) good resolution due to column selectivity. [From (6), used with permission.]

4. Capacity Factor. The capacity or retention factor, k', is a measure of the amount of time a chromatographed species (solute) spends in/on the stationary phase relative to the mobile phase. The relationship between capacity factors and chromatographic retention (which may be expressed in units of either volume or time, as previously discussed in section 31.3.4.3.2), is shown below:

$$k' = \frac{KV_s}{V_m} = \frac{V_R - V_m}{V_m} = \frac{t_R - t_o}{t_o} \qquad [10]$$

where:

k' = capacity factor
K = distribution coefficient of the solute
V_s = volume of stationary phase in column
V_m = volume of mobile phase
V_R = retention volume of solute
t_R = retention time of solute
t_o = retention time of unretained components (solvent front)

Small values of k' indicate little retention, and components will be eluted close to the solvent front, resulting in poor separations. Large values of k' result in improved separation but also can lead to broad peaks

and long analysis times. On a practical basis, k' values within the range of 1-15 are generally used. (In the equation for R_s, k' is actually the average of k_1' and k_2' for the two components separated.)

31.3.4.3.4 Quantitative Analysis Assuming that good chromatographic resolution and identification of sample components have been achieved, quantitation involves measuring peak height, area, or mass and comparing these data with those for standards of known concentration. (It should be remembered that a completely resolved peak is not necessarily equivalent to a pure substance. One peak can represent several components that are not resolvable under the chromatographic conditions utilized.)

1. **Peak Height versus Peak Area.** There is much debate about which of these techniques is more useful. In general, peak height is less dependent on flow rate, but peak area is less affected by other instrumental and operator variations. Peak height is simply measured as the distance from baseline to peak maximum (see Fig. 31-11). Interpolation of the baseline from start to finish may be used to compensate for baseline drift (often a problem in gradient elution). Peak height measurement generally gives precisions of 1-2% and should not be used with visibly distorted peaks.

Several methods may be used to measure peak area, as depicted in Fig. 31-13. Since chromatographic peaks often approximate a triangle, area can be calculated by the formula for a triangle, $A = (w/2)h$ (Fig. 31-13a). The width at half-height (w_h) is used to reduce errors due to adsorption and tailing. This method should be used only for symmetrical peaks or those that have similar shapes. The area measured is less than the true area but is proportional to sample size, provided peaks are not badly distorted. Precision depends on the ratio of $h:w_h$, which preferably falls into a range of 2-10. A second area-measurement method is triangulation, which requires drawing lines tangent to the sides of the peak. Peak height and width are then measured as shown in Fig. 31-13b. Since this technique offers no greater accuracy than the previous method and is subject to operator error (drawing of the tangent lines), it is not recommended. A planimeter is a mechanical device that can be used to measure peak areas by tracing around their perimeters (Fig. 31-13c). The precision and accuracy of this method depend on the device itself and on operator skill. The planimeter technique can be more accurate than triangulation, especially if the peaks are skewed.

The cut-and-weigh method of quantitating peak mass requires carefully cutting out the chromatographic peak (or a copy of it) and weighing the paper on an analytical balance. The inaccuracy of cutting can be minimized by keeping the ratio of $h:w_h$ between 1 and 10. Homogeneity of the paper, moisture content, and the weight of the paper all are important factors.
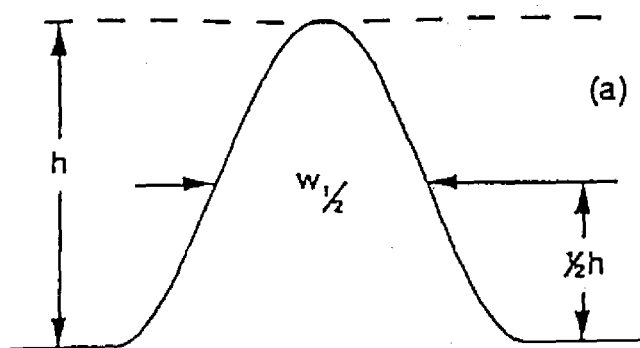
Height Times Width at Half-Height

Triangulation

Planimetry

but this method is superior to triangulation techniques for irregularly shaped peaks.

Electronic integrators provide the chromatographer with highly precise and automatic conversion of detector output into numerical form. The only disadvantages of this method are that digital integrators are rather expensive and setting parameters often requires a fairly high level of operator sophistication.

2. External versus Internal Standards. Having quantitated sample peaks, one must compare these data with appropriate standards of known concentration to determine sample concentrations. Comparisons may be by means of external or internal standards. Comparison of peak height, area, or mass of unknown samples with standards injected separately, i.e., external standards, is common practice. Standard solutions covering the desired concentration range (preferably diluted from one stock solution) are chromatographed, and the appropriate data (peak height, area, or mass) plotted versus concentration to obtain a standard curve. An identical volume of sample is then chromatographed, and height, area, or mass of the sample peak is used to determine sample concentration via the standard curve (Fig. 31-14a). This absolute calibration method requires precise analytical technique and

Three different methods of estimating peak area. (A) Peak height × width at half-height; (B) triangulation; (C) planimetry. [From (6), used with permission.]
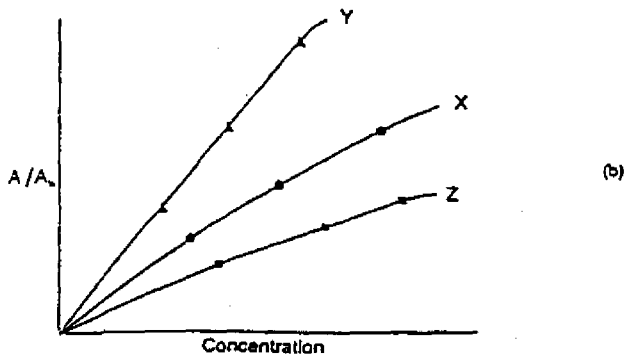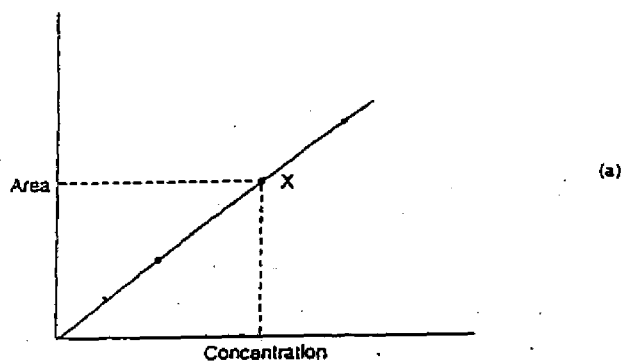
Calibration curves for quantitation of a sample component, x. (a) External standard technique; (b) internal standard technique. [Adapted from (6), with permission.]

requires that detector sensitivity be constant from day to day if the calibration curve is to remain valid.

Use of the internal standard (relative or indirect) method can minimize errors due to sample preparation, apparatus, and operator technique. In this technique, a compound is utilized that is structurally related to, but is eluted independently of, compounds of interest in the sample to be analyzed. Basically, the amount of each component in the sample is determined by comparing the height, area, or mass of that component peak to the height, area, or mass of the internal standard peak. However, variation in detector response between compounds of different chemical structure must be taken into account. One way to do this is by first preparing a set of standard solutions containing varying concentrations of the compound(s) of interest. Each of these solutions is made to contain a known and constant amount of the internal standard. These standard solutions are chromatographed, and peak height, area, or mass is measured. Ratios of peak height, area, or mass (compound of interest/internal standard) are calculated and plotted against concentration to obtain calibration curves such as those shown in Fig. 31-14b. A separate response curve must be plotted for each sample component to be quantitated. Next, a known amount of internal standard is added to the unknown sample, and the sample is chromatographed. Peak height, area, or mass ratios (compound of interest/internal standard) are calculated and used to read the concentration of each relevant component from the appropriate calibration curve. The advantages of using internal standards are that injection volumes need not be accurately measured and the detector response need not remain constant since any change will not alter ratios. The main disadvantage of the internal standard technique is the difficulty of finding a standard that does not interfere chromatographically with components of interest in the sample.

31.3.4.4 Supercritical Fluid Chromatography

The technique of supercritical fluid chromatography (SFC) was initially demonstrated more than 30 years ago. Since the 1960s, however, more effort has been put into the development of HPLC. Now that HPLC has reached maturity, there is increasing interest in SFC.

A supercritical fluid is one that is above both its critical pressure (P_c) and critical temperature (T_c). (The combination of P_c and T_c is known as the critical point.) A supercritical fluid can be formed from a conventional gas by increasing the pressure, or from a conventional liquid by raising the temperature. Commonly used fluids, such as carbon dioxide, are purchased as liquefied gases. Carbon dioxide frequently is used as a mobile phase for SFC; however, it is not a good solvent for polar and high-molecular-weight compounds. A small amount of a polar, organic

solvent such as methanol can be added to a nonpolar supercritical fluid to enhance solute solubility, improve peak shape, and alter selectivity. (Such a mobile phase modifier may be referred to as an entrainer.) Other supercritical fluids that have been used in food applications include nitrous oxide, trifluoromethane, sulfur hexafluoride, pentane, and ammonia.

When employed as mobile phases, supercritical fluids confer chromatographic properties intermediate to liquid and gas chromatography. The high diffusivity and low viscosity of supercritical fluids mean decreased analysis times and improved resolution compared to liquid chromatography. Supercritical fluid chromatography offers a wide range of selectivity adjustment, since k' and α (section 31.3.4.3.3) may be altered by changes in pressure and temperature as well as changes in mobile phase composition and the stationary phase. In addition, SFC makes possible the separation of nonvolatile, thermally labile compounds that are not amenable to gas chromatography.

Supercritical fluid chromatography can be performed using either packed columns or capillaries (each requires appropriately designed instrumentation). In the case of the former, column packing materials are similar to those used for HPLC. Small particle, porous, high surface area, hydrated silica may serve as the stationary phase itself, or simply as a support for a bonded stationary phase (Chapter 32). Polymer-based packings have been used, but are less satisfactory owing to long solute retention times. Capillaries are generally coated with a polysiloxane (—Si—O—Si) film, which is then crosslinked to form a polymeric stationary phase that cannot be washed off by the mobile phase. Polysiloxanes containing different functional groups, such as methyl, phenyl, or cyano, may be used to vary the polarity of this stationary phase. Instrumentation for (packed column) SFC is similar to that used for HPLC with one major difference. A back pressure regulator is used to control the outlet pressure of the system. (Without this device, the fluid would expand to a low pressure, low density gas.) Besides the advantages of deceased analysis time and improved resolution, SFC offers the possibility to use a wide variety of detectors, including those designed for gas chromatography.

Supercritical fluid chromatography has been used primarily for nonpolar compounds. Chester et al. (3) review recent applications of SFC. Fats, oils, and other lipids are compounds to which SFC is increasingly applied. The noncaloric fat substitute, olestra, was characterized by SFC-MS (mass spectroscopy). Other researchers have used SFC to detect pesticide residues, study thermally labile compounds from members of the *Allium* genus, fractionate citrus essential oils, and characterize compounds extracted from microwave packaging (3). Borch-Jensen and Mollerup (2) discussed the use of packed column and capillary SFC for the analy-

sis of food and natural products, especially fatty acids and their derivatives, glycerides, waxes, sterols, fat-soluble vitamins, carotenoids, and phospholipids. Results obtained by SFC are compared to those from GC and HPLC.

31.4 SUMMARY

Chromatography is a separation method based on the partitioning of a solute between a moving, or mobile, phase and a fixed, or stationary, phase. The mobile phase may be liquid, gas, or a supercritical fluid. The stationary phase may be a liquid or a solid. This chapter focuses primarily on liquid chromatography, chromatography performed (at atmospheric pressure) with a liquid mobile phase. Basic physicochemical principles underlying all liquid chromatographic separations are adsorption, partition, ion-exchange, size-exclusion, and affinity. General terminology, and the specific techniques of paper, thin-layer, and column liquid chromatography are described. In the latter, sample components may be resolved into separate bands or peaks as they migrate through a column packed with stationary phase. Factors that contribute to chromatographic resolution are column efficiency, selectivity, and capacity. A chromatogram provides both qualitative and quantitative information via retention time (or volume) and peak height (area or mass) data.

For an introduction to the techniques of HPLC and GC the reader is referred again to Chapters 32 and 33 in this text. The book by R.M. Smith (10) contains information on basic concepts of chromatography and chapters devoted to thin-layer, liquid, and high performance liquid chromatography, as well as an extensive discussion of gas–liquid chromatography. In addition to a bibliography at the end of each chapter, this well-written volume also contains an appendix that describes the chromatographic literature, including references to specific applications such as food. Books by Touchstone (12) and Berger (1) contain detailed information on thin-layer and supercritical fluid chromatography, respectively. *Chromatography*, the standard work edited by E. Heftmann (1992 and earlier editions), is an excellent source of information on both fundamentals (Part A) and applications (Part B) of chromatography. Part B includes chapters on the chromatographic analysis of amino acids, proteins, lipids, carbohydrates, and phenolic compounds. In addition, *Fundamental* and *Applications Reviews* published (in alternating years) by the journal *Analytical Chemistry* relate new developments in all branches of chromatography, as well as their application to specific areas such as food. Recent books and general review papers are referenced, along with research articles published during the specified review period.

31.5 STUDY QUESTIONS

1. Differentiate batch, continuous, and countercurrent extraction, and explain how extraction relates to chromatography.
2. Describe the difference between adsorption and partition chromatography with respect to stationary phases. How does a solute interact with the stationary and mobile phase in each case?
3. Distinguish between normal-phase and reversed-phase chromatography by comparing the nature of the stationary and mobile phases and the order of solute elution.
4. What is the advantage of bonded supports over coated supports for partition chromatography?
5. You applied a mixture of proteins, in a buffer at pH 8.0, to an anion-exchange column. On the basis of some assays you performed, you know that the protein of interest adsorbed to the column.
   a. Does the anion-exchange stationary phase have a positive or negative charge?
   b. What is the overall charge of the protein of interest that adsorbed to the stationary phase?
   c. Is the isoelectric point of the protein of interest (adsorbed to the column) higher or lower than pH 8.0?
   d. What are the two most common methods you could use to elute the protein of interest from the anion-exchange column? Explain how each method works. (See also Chapter 16.)
6. Explain how you would use size-exclusion chromatography to estimate the molecular weight of a protein molecule. Include an explanation of what information must be collected and how it is used.
7. Would you use a polystyrene- or a polysaccharide-based stationary phase for work with proteins? Explain you answer.
8. Explain the principle of affinity chromatography, why a spacer arm is used, and how the solute can be eluted.
9. Compare paper chromatography, thin-layer chromatography, and column liquid chromatography. Explain similarities and differences.
10. What is gradient elution from a column, and why is it often advantageous over isocratic elution?
11. Using the Van Deemter equation, HETP, and N, as appropriate, explain why the following changes may increase the efficiency of separation in column chromatography:
    a. changing the flow rate of the mobile phase
    b. increasing the length of the column
    c. reducing the inner diameter of the column
12. How can chromatographic data be used to quantitate sample components?
13. Why would you choose to use an internal standard rather than an external standard? Describe how you would select an internal standard for use.
14. Explain how supercritical fluid chromatography (SFC) differs from liquid chromatography, including the advantages of SFC.

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High Performance Liquid Chromatography

Mary Ann Rounds and Jesse F. Gregory, III

32.1 INTRODUCTION

High performance liquid chromatography (HPLC) developed during the 1960s as a direct offshoot of classical column liquid chromatography through improvements in the technology of columns and instrumental components (pumps, injection valves, and detectors). Originally, HPLC was the acronym for *high pressure liquid chromatography*, reflecting the high operating pressures generated by early columns. By the late 1970s, however, *high performance liquid chromatography* had become the preferred term, emphasizing the effective separations achieved. In fact, newer columns and packing materials offer high performance at moderate pressure (although still high relative to gravity-flow liquid chromatography). *Advantages* of HPLC over traditional low pressure column liquid chromatography include:

1. Speed (many analyses can be accomplished in 30 min or less).
2. Improved resolution (due in part, to a wide variety of stationary phases)
3. Greater sensitivity (various detectors can be employed).
4. Reusable columns (although initially expensive, columns can be used for many analyses).
5. Ideal for ionic species and large molecules (substances of low volatility)
6. Easy sample recovery

High performance liquid chromatography can be applied to the analysis of any compound with solubility in a liquid that can be used as the mobile phase. Unlike gas chromatography, sample components need not be volatile; derivatization, when used with HPLC, serves to enhance detectability of the analyte. Although most frequently employed as an analytical technique, HPLC also may be used in the preparative mode. (Preparative chromatography is utilized to obtain highly purified compounds in quantities ranging from milligrams to kilograms.)

Applications of HPLC to the analysis of food materials began in the late 1960s, and its use soared with the development of column packing materials that would separate sugars. Using HPLC to analyze sugars was justified economically as a result of sugar price increases in the mid-1970s which motivated soft-drink manufacturers to substitute high-fructose corn syrup for sugar. Monitoring sweetener content by HPLC assured a good quality product. Other early food applications included the analysis of pesticide residues in fruits and vegetables, organic acids, lipids, amino acids, toxins (such as aflatoxins in peanuts), and vitamins (1). High performance liquid chromatography

continues to be applied to these, and many more, food-related analyses today (2–4).

32.2 COMPONENTS OF AN HPLC SYSTEM

A schematic diagram of a basic HPLC system is shown in Fig. 32-1. The main components of this system—pump, injector, column, detector, and recorder/integrator/data system—are discussed briefly in the sections below. (A fraction collector is added only if further analysis or purification of separated components is needed.) Connecting tubing, tube fittings, and the materials out of which components are constructed also are important because they influence system performance and lifetime. References (1) and (5–7) include detailed discussions of HPLC equipment, with the books by Bidlingmeyer (1) and Smith (5) especially appropriate for beginners. Two useful books on HPLC troubleshooting are those written by Gertz (8) and Dolan and Snyder (9). In addition, much information on HPLC equipment, hardware, and troubleshooting hints may be found in publications such as *LC·GC*, *American Laboratory*, *Chemical & Engineering News*, and similar periodicals. Manufacturers are also a source of practical information on HPLC instrumentation and hardware.

32.2.1 Pump

The role of the HPLC pump is to deliver the mobile phase through the system, typically at a flow rate of 1 ml/min, in a controlled, accurate, and precise manner. The two main types of pumps used are constant pressure and constant volume. Constant pressure pumps may be either of a reciprocating or a syringe-type design. Reciprocating pumps produce a pulsating flow, thereby requiring the addition of mechanical or electronic pulse dampers to suppress fluctuations. A mechanical pulse damper or dampener consists of a device (such as a deformable metal component or tubing filled with compressible liquid) that can change its volume in response to changes in pressure. Screw-driven syringe pumps produce pulseless flow but suffer the disadvantage of limited reservoir capacity. Gradient elution systems for HPLC may utilize low-pressure mixing, in which mobile phase components are mixed before entering the high pressure pump, or high-pressure mixing, in which two or more independent, programmable pumps are used.

Most commercially available HPLC pumping systems and connecting lines are made of grade ANSI 316 stainless steel, which can withstand the pressures generated and is resistant to corrosion by oxidizing agents, acids, bases, and organic solvents. Mineral acids and halide ions do attack stainless steel. Thus, the system
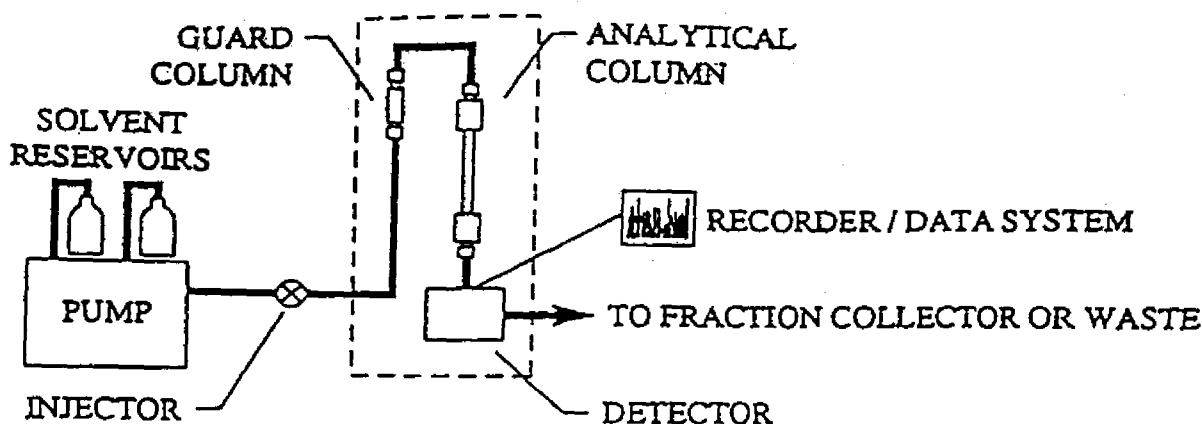
32-1 Schematic representation of a system for high performance liquid chromatography (not drawn to scale). Column(s)
figure and detector may be thermostatted, as indicated by the dashed line, for operation at elevated temperature.

should be rinsed thoroughly with water if these sub-
stances have been used in the mobile phase. All HPLC
pumps contain moving parts such as check valves and
pistons, and are quite sensitive to dust and particulate
matter in the liquid being pumped. Therefore, it is
advisable to filter the mobile phase using 0.45 or 0.22
μm porosity filters prior to use. Degassing HPLC elu-
ents, by application of a vacuum and/or ultrasonica-
tion or by sparging with helium, also is recommended
to prevent the problems that can be caused by air bub-
bles in a pump or detector.

32.2.2 Injector

The role of the injector is to place the sample into the
flowing mobile phase for introduction onto the col-
umn. Originally, injection onto the HPLC column was
made through a septum, similar to the method used in
gas chromatography. Nowadays, virtually all HPLC
systems use valve injectors, which separate sample
introduction from the high-pressure eluent system.
With the injection valve in the LOAD position (Fig. 32-
2A), the sample is loaded, via syringe, into an external,
fixed-volume loop at atmospheric pressure. Eluent,
meanwhile, flows directly from the pump to the col-
umn at high pressure. When the valve is rotated to the
INJECT position (Fig. 32-2B), the loop becomes part of
the eluent flow stream and sample is carried onto the
column.

Such injectors are generally trouble free and afford
good precision. Changing the loop allows different vol-
umes to be injected. Although injection volumes of
10–100 μl are typical, both larger (e.g., 1–10 ml) and
smaller (e.g., ≤2 μl) sample volumes can be loaded by
utilizing special hardware. Volumes as small as 20 nl
can be injected by utilizing an internal loop; the
injected volume is that of the connecting channel in the

valve rotor. An important advantage of the loop valve
design is that it is readily adapted to automatic opera-
tion. Thus, automated sample injectors, or autosam-
plers, may be used to store and inject large numbers of
samples. Samples are placed in uniform-size vials,
sealed with a septum, and held in a (possibly refriger-
ated) tray. A needle penetrates the septum to withdraw
solution from the vial, and an electronically or pneu-
matically operated valve introduces it onto the col-
umn. Autosamplers can reduce the tedium and labor
costs associated with routine HPLC analyses and
improve assay precision. However, considerable time
must be invested in initial setup, and these devices are
seldom trouble free. Because samples may remain
unattended for 12–24 hr prior to automatic injection,
sample stability is a key factor to consider before pur-
chasing this accessory.

32.2.3 Column

An HPLC column could be considered a tool for the
separation of molecules. Since both external hardware
and internal packing material are important, these two
topics are discussed separately.

32.2.3.1 Column Hardware

An HPLC column is usually constructed of stainless
steel tubing with terminators that allow it to be con-
nected between the injector and detector of the system
(Fig. 32-1). Columns also have been made from glass,
fused silica, titanium, and PEEK (polyether ether ke-
tone) resin. Many types and sizes are commercially
available, ranging from 5 cm x 50 cm (or larger) prepar-
ative columns down to wall-coated capillary columns.

32.2.3.1.1 Precolumns Auxillary columns that pre-
cede the analytical HPLC column may be termed pre-

A

B
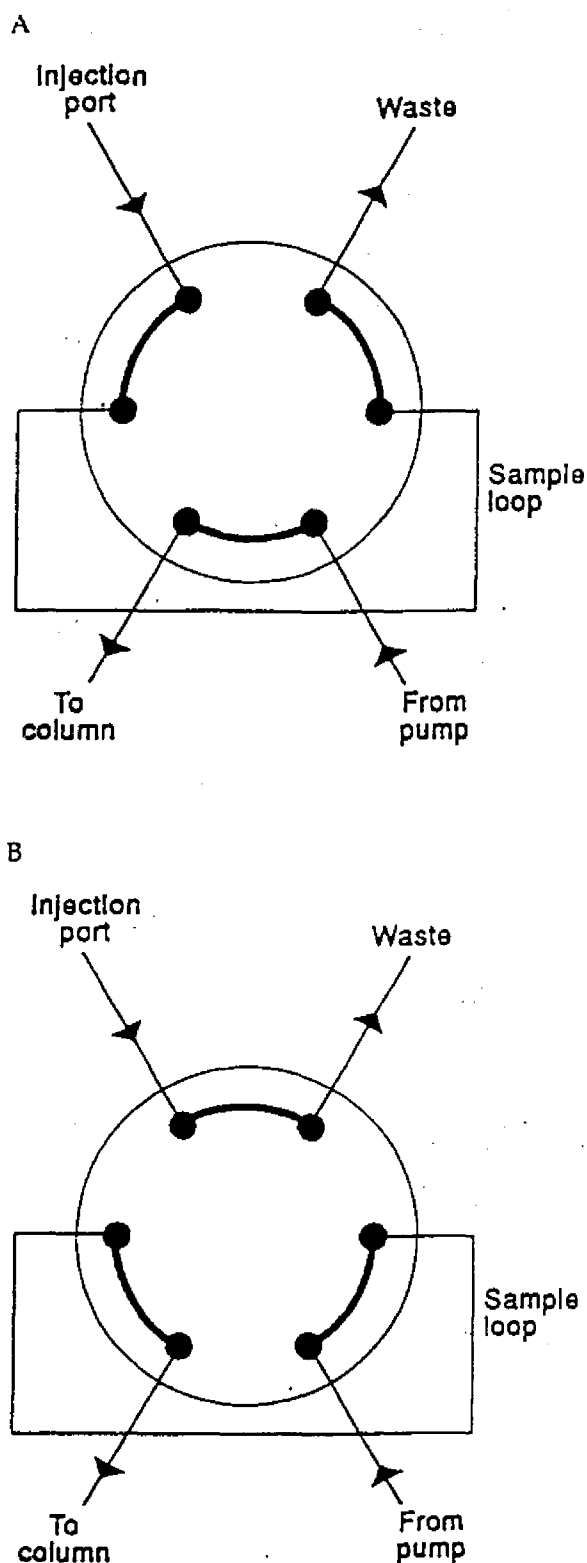
| 32-2 |
| figure |

Valve-type injector. The valve allows the sample loop to be (A) isolated from the pump eluent stream (LOAD position) or (B) positioned in it (INJECT position). [From (6), used with permission.]

columns. Presaturator columns containing large particles of bare silica may be placed between the HPLC pump and injector to presaturate the mobile phase with silica. This can help to slow down dissolution of silica-based packing materials during the use of aqueous mobile phases.

Short (≤ 5 cm) expendable columns, called guard columns, often are used to protect the analytical column from strongly adsorbed sample components. A guard column (or cartridge) is installed between the injector and analytical column via short lengths of capillary tubing (or a cartridge holder). Usually of the same internal diameter as the analytical column, guard columns are designed with relatively small total volumes and minimal dead volumes, so that they do not cause significant band broadening. They may be filled with either pellicular media (see section 32.2.3.2.2) of the same bonded phase as the analytical column, or with microparticulate (≤ 10 μm) packing material identical to that of the analytical column. A guard column (or cartridge) should be repacked or replaced before its binding capacity is exceeded and contaminants break through to the analytical column. Guard columns containing large pellicular particles, e.g. 35–40 μm, may be dry-packed by the user. Microparticulate media, which must be slurry packed (as discussed in section 32.2.3.3), often can be purchased as prepacked, disposable inserts for use in a special holder (1).

32.2.3.1.2 Analytical Columns The most commonly used analytical HPLC columns are 10, 15, or 25 cm long with an internal diameter of 4.6 or 5 mm (5, 6). (Optimum column length is dictated by the number of theoretical plates needed to give the desired separation, as discussed in Chapter 31, section 31.3.4.3.3.) Analytical columns are generally packed with 3, 5, or 10 μm particles and are operated at flow rates of 1–2 ml/min. Short (3 cm) columns, packed with ≤ 3 μm particles, are gaining popularity for fast separations, for example, in method development or process monitoring. In recent years, the use of columns with smaller internal diameters has increased. The advantages of using smaller diameter columns include (10):

1. Decreased consumption of both mobile and stationary phases
2. Decreased peak volume (which leads to increased peak concentration and increased detection sensitivity)
3. Increased resolution (with long columns)
4. Applicability to temperature programming
5. Reduced equilibration time
6. Possibility to couple HPLC with mass spectrometry (MS)

Various names have been used for the reduced-volume

columns. Dorsey et al. (11) refer to columns with internal diameters of 0.5–2.0 mm as microbore, while open tubular or packed columns having internal diameters of <0.5 mm are termed microcolumns. (Open tubular columns consist of a capillary, the inner surface of which is coated with a thin layer of stationary phase.) To achieve good performance from microcolumns, it is essential to have an HPLC system with very low dead-volume, so that peak broadening outside the column does not destroy resolution achieved within the column. Pumps and injectors designed specifically for use with these columns are available from commercial suppliers.

32.2.3.2 HPLC Column Packing Materials

The development of a wide variety of column packing materials has contributed substantially to the success and widespread use of HPLC.

32.2.3.2.1 General Requirements A packing material serves, first of all, to form the chromatographic bed. It may or may not be involved in the actual separation process, i.e., distribution of a solute between two phases. In classical liquid–liquid chromatography, the packing serves only to support the stationary phase, which is the liquid that resides in its pores (Chapter 31, section 31.3.3.2). In size-exclusion chromatography, it is important that there be no interaction between solutes and the column packing material, since separation is accomplished, ideally, on the basis of differences in molecular size (Chapter 31, section 31.3.3.4). However, in adsorptive modes of chromatography, including ion-exchange and affinity, the column packing material simultaneously serves as both support and stationary phase. Additional requirements for HPLC column packing materials are (7):

1. Availability in a well-defined particle size, with a narrow particle size distribution
2. Sufficient mechanical strength to withstand pressure generated during packing and use
3. Good chemical stability

32.2.3.2.2 Silica-Based Column Packings 1. Porous Silica. Porous silica meets the above criteria quite well and can be prepared in a wide range of particle and pore sizes (with a narrow particle size distribution). Both particle size and pore diameter are important with regard to HPLC separations. Small particles reduce the distance a solute must travel between stationary and mobile phases. This facilitates equilibration and results in good column efficiencies, i.e. a large number of theoretical plates per unit of column length (Chapter 31, section 31.3.4.3.3). However, small particles also mean greater flow resistance; thus, higher

pressure drops at equivalent flow velocity. Spherical particles of 3, 5, or 10 μm diameter are utilized in analytical columns. Larger (sometimes irregularly shaped) particles may be used for industrial-scale preparative chromatography, since they are less expensive.

One half or more of the volume of porous silica consists of voids or pores (7). Choice of pore diameter is important, inasmuch as packing material surface area is inversely related to the mean pore diameter. In adsorptive modes, chromatographic retention (Chapter 31, section 31.3.4.3.2) increases as the amount of stationary phase surface area increases. Thus, use of the smallest possible pore diameter will maximize surface area and sample capacity (the amount of sample that can be separated on a given column). Packing materials with a pore diameter of 50–100 Å and surface area of 200–400 m²/g are used for low-molecular-weight (<500) solutes. For increasingly larger molecules, such as proteins and nucleic acids, it is necessary to use wider pore materials (pore diameter ≥ 300 Å) so that internal surface is accessible to the solute (7).

Silica consists mainly of silicon dioxide, SiO_2, with each Si atom at the center of a tetrahedron. On the surface, one remaining valency is generally occupied by an —OH group, referred to as a silanol. These weakly acidic groups ($pK_a \sim 9$) (12) are rather reactive and can be utilized to modify the silica surface.

2. Bonded Phases. So-called bonded phases (Fig. 32-3A) are made by covalently bonding hydrocarbon groups to the surface of silica particles via surface silanols (12, 13). Often, the silica is reacted with an organochlorosilane:

$$\overset{\backslash}{\underset{/}{-Si}}-OH + Cl-\overset{\overset{R_1}{|}}{\underset{\underset{R_2}{|}}{Si}}-R_3 \longrightarrow \overset{\backslash}{\underset{/}{-Si}}-O-\overset{\overset{R_1}{|}}{\underset{\underset{R_2}{|}}{Si}}-R_3 + HCl \quad [1]$$
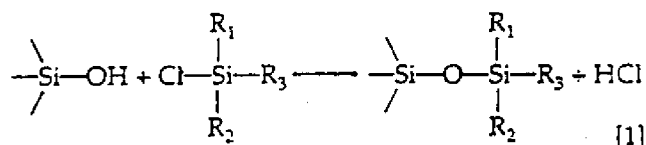
Substituents R_1 and R_2 may be halides or methyl groups. The nature of R_3 determines whether the resulting bonded phase will exhibit normal-phase, reversed-phase, or ion-exchange chromatographic behavior. The siloxane (—Si—O—Si—) bond is stable in the pH range ca. 2–7.5. Chiral selectors, for the resolution of enantiomers, such as enantiomeric lipids, also may be attached via silica surface modifications (7).

The main disadvantage of silica and silica-based, bonded-phase column packings is that the silica skeleton slowly dissolves in aqueous solutions, especially at pH > 8. Consequently, much effort has gone into the development of nonsilica HPLC packings. Other inorganic materials, of greater pH stability, that may be used are alumina, zirconia, titania, porous graphitic carbon, and hydroxyapatite.

3. Pellicular Packings. A pellicular packing material (Fig. 32-3B) is made by depositing a thin layer or

A ⎯ Bonded Phase
B ⎯ Thin Surface Coating or Layer

Silica
Inert core

C ⎯ Micropores
D ⎯ Micropores
⎯ Macropores
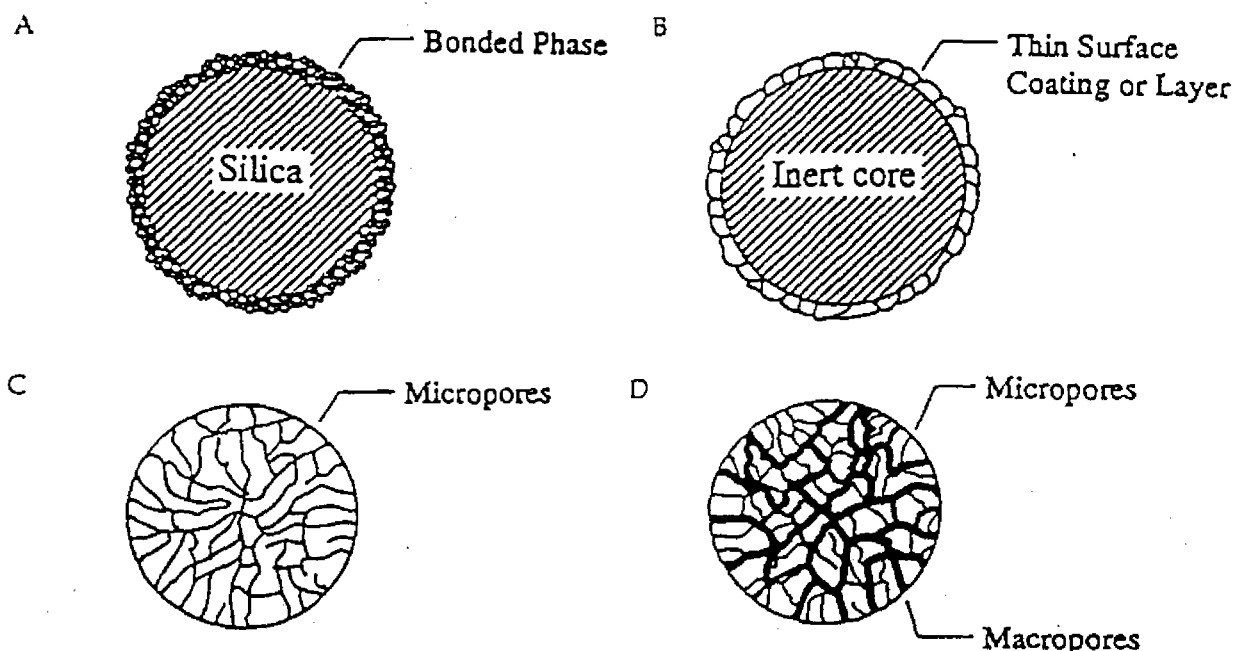
32-3 figure Some types of packing materials utilized in HPLC. (A) Bonded-phase silica; (B) pellicular packing; (C) microporous polymeric resin; (D) macroporous polymeric resin. [Adapted from (13), p. 621 by courtesy of Marcel Dekker, Inc.]

coating onto the surface of an inert, usually nonporous, microparticulate core. Core material may be either inorganic, such as silica, or organic, such as poly-(styrene-divinylbenzene) or latex. Functional groups such as ion-exchange sites are then present at the surface only. The rigid core ensures good physical strength whereas the thin stationary phase provides for rapid mass transfer and favorable column efficiency. A disadvantage is that the thin surface coating limits the number of interactive sites; consequently, binding capacity is low. Glass beads of limited porosity covered with chemically attached groups and silicas with extensive bonded-phase coverage also exhibit characteristics of pellicular packings. The distinction between bonded phase and pellicular packings is not always clear cut (13).

Pellicular coatings on porous supports, such as macroporous silica or polystyrene, have proven to be quite useful for HPLC of large biological molecules such as proteins and oligonucleotides. A polyamine is physically adsorbed to the support surface, and then crosslinked into a permanent polymeric layer. The resulting pellicular coating extends pH stability of underlying inorganic media and can mask the undesirable hydrophobicity of a polystyrene matrix (14).

32.2.3.2.3 Polymeric Column Packings. Synthetic organic resins offer the advantages of good chemical stability and the possibility to vary interactive properties through direct chemical modification. Two major

categories of polymeric packing materials exist. Other novelty types, such as porous polymer rods, have been introduced for use in preparative chromatography but will not be discussed here.

1. Microporous (Microreticular). Microporous or gel-type resins (Fig. 32-3C) are comprised of crosslinked copolymers in which the apparent porosity, evident only when the gel is in its swollen state, is determined by the degree of crosslinking. Styrene, crosslinked with 2–16% divinylbenzene, is an example of a microporous polymer. These gel-type packings undergo swelling and contraction with changes in the chromatographic mobile phase, which can result in bead fracture, poor mass transfer, and increased pressure drop and resistance to flow. Microporous polymers of less than ca. 8% crosslinking are not sufficiently rigid for HPLC use.

2. Macroporous (Macroreticular). Macroporous resins are highly crosslinked (e.g., ≥50%) and consist of a network of microspheric gel beads joined together to form a larger bead (Fig. 32-3D). Large, permanent pores, ranging from 100 to 4000Å or more in diameter, and high surface areas (≥100 m²/g) are the result of interstitial spaces between the microbeads (13). Rigid microparticulate poly(styrene-divinylbenzene) packing materials of the macroporous type are popular for HPLC use. They are stable from pH 1 to 14 and are available in a variety of particle and pore sizes. These resins can be used in unmodified form for reversed-phase chromatography or functionalized for use in

other HPLC modes. Methacrylate polymers also are used, and can be made sufficiently hydrophilic for use with proteins.

32.2.3.3 Column Packing Procedures

The column packing process serves to arrange packing material particles in a bed of high regularity and stability. It is essential that the bed be uniform across the width of the column if low plate heights (Chapter 31, section 31.3.4.3.3) are to be obtained. Techniques such as sedimentation, dry packing with tamping, or just pouring the material into the column are not successful with particles of <20 μm diameter. Thus, slurry packing is virtually always used in HPLC. The packing material is suspended in a suitable liquid, such as an alcohol–chloroform mixture, and this slurry is pumped into the column (via a "displacer" liquid) at a rather high flow rate and pressure. The column bed is formed by autofiltration of the particles. Many variables must be carefully controlled and column packing is probably the least understood aspect of HPLC. (Recipes in use are often based on experience rather than theory.) Since many companies market well-packed columns, it is generally unwise for a laboratory to undertake a column packing project (7).

32.2.4 Detector

A detector translates concentration changes in the HPLC column effluent into electrical signals. Spectrochemical, electrochemical, or other properties of solutes may be measured by a variety of instruments, each of which has advantages and disadvantages. The choice of which to use depends on solute type and concentration, and on detector sensitivity, linear range, and compatibility with the solvent and elution mode to be used. Economic factors, i.e., initial and operating costs, also may influence detector selection.

The most widely used HPLC detectors are based on ultraviolet-visible (UV-Vis) and fluorescence spectrophotometry, refractive index determination, and electrochemical analysis. (See Chapter 26 for detailed discussion of UV-Vis and fluorescence spectrophotometry.) Many other methods, such as light scattering or spectrometry, can be applied to the detection of analytes in HPLC eluents. More than one type of HPLC detector may be used series, to provide increased specificity and sensitivity for multiple types of analytes. In one food-related application, a multidetector HPLC system equipped with a diode array absorption detector coupled to fluorescence and electrochemical detectors was used to monitor a wide variety of Maillard reaction products (such as hydroxymethylfurfural) and follow their kinetics (2).

32.2.4.1 UV-Visible Absorption Detectors

Many HPLC analyses are carried out using the UV-Vis absorption detector, which can measure the absorption of radiation by chromophore-containing compounds. (These include simple unsaturated species, such as ketones, conjugated and aromatic compounds, and a number of inorganic ions and complexes.) As long as monochromatic light is used, the magnitude of the absorption signal is directly proportional to analyte concentration in accordance with the Beer's law:

Absorbance = ε × cell path length
× molar sample concentration [2]

Absorbance is directly dependent on the molar absorptivity, ε, at the wavelength of detection. Thus, detector sensitivity and response will differ for analytes, depending on their chromophores.

The three main types of UV-Vis absorption detectors are fixed wavelength, variable-wavelength, and diode array spectrophotometers (5, 6). As its name implies, the most simple design operates at a single, fixed wavelength. A filter is used to isolate a single emission line, e.g., at 254 nm, from a source such as a mercury lamp. This type of detector is easy to operate and inexpensive, but of limited utility.

The most popular general purpose HPLC detector today is the variable-wavelength detector in which deuterium and tungsten lamps serve as sources of ultraviolet and visible radiation, respectively. Wavelength selection is provided by a monochromator, a device that acts somewhat like a prism to deflect light. An exit slit in the monochromator allows light from a limited range of wavelengths to pass through, and rotating the monochromator allows one to change the operating wavelength.

Diode array spectrophotometric detectors can provide much more information about sample composition than is possible with monochromatic detection. In this instrument, all the light from a deuterium lamp is spread out into a spectrum that falls across an array of photodiodes mounted on a silicon chip. These can be read virtually simultaneously by a microcomputer to provide the full absorption spectrum from 200 to 700 nm every 0.1 sec. A dedicated computer, usually supplied with the detector, is needed to handle the large amount of data generated. Photodiode array detectors may enable the components of a mixture to be identified, and can be used to assess purity (differences in the absorption spectrum between front and tailing edges of a peak indicate an unresolved impurity). Considerably more expensive than variable-wavelength detectors, they are useful in method development and in routine analysis in which additional evidence of peak identity is needed.

32.2.4.2 Fluorescence Detectors

Some organic compounds can reemit a portion of absorbed UV-visible radiation at a longer wavelength (lower energy). This is known as fluorescence, and measurement of the emitted light provides another useful detection method. Fluorescence detection is both selective and very sensitive, providing 100- to 1000-fold lower detection limits than for the same compound in absorbance spectrophotometry. Although relatively few compounds are inherently fluorescent, analytes often can be converted into fluorescent derivatives (see section 32.2.4.7). Background noise introduced by extraneous radiation is the main limitation of fluorescence detectors. Ideal for trace analysis, fluorescence detection has been used for the determination of various vitamins in foods and supplements, monitoring aflatoxins in stored cereal products, and the detection of polynuclear aromatic hydrocarbons in wastewater (5).

32.2.4.3 Refractive Index Detectors

Refractive index (RI) detectors measure change in the refractive index of the mobile phase due to dissolved analytes. This provides a nearly universal method of detection. However, because a bulk property of the eluent is being measured, refractive index detectors are less sensitive than the more specific types. Peaks may be positive or negative, depending an analyte refractive index relative to that of the eluent, and these detectors are more sensitive to changes in ambient temperature than other types. Another disadvantage is that they cannot be used with gradient elution, as any change in eluent composition will alter its refractive index, thereby changing the baseline signal. Refractive index detectors are widely used for analytes that do not contain UV-absorbing chromophores, such as carbohydrates and lipids.

32.2.4.4 Electrochemical Detectors

Two electroanalytical methods used for HPLC detection are based either on electrochemical oxidation–reduction of the analyte or on changes in conductivity of the eluent. Amperometric detectors measure the change in current as analyte is oxidized or reduced by the application of voltage across electrodes of the flow cell. This method is highly selective (nonreactive compounds give no response) and very sensitive, often 10^4 better than ultraviolet spectrophotometric detection (5). A major application of electrochemical detection has been for the routine determination of catecholamines, phenolic compounds of clinical importance that are present in blood and tissues at very low levels. The development of a triple-pulsed amperometric detector

that overcame the problem of electrode poisoning (accumulation of oxidized product on the electrode surface) has allowed electrochemical detection to be applied to the analysis of carbohydrates (see section 32.3.3.2.2). Pulsed electrochemical detection also has been shown to have excellent sensitivity for the quantification of flavor-active alcohols, particularly terpenols (2).

Analytes that are ionized and can carry a charge can be detected by measuring the change in eluent conductivity between two electrodes. Conductometric (or conductivity) detection has been used mainly to detect inorganic anions and cations and organic acids upon elution from weak ion-exchange columns. Its principal application has been as the basis of ion chromatography (section 32.3.3.2.1).

32.2.4.5 Other HPLC Detectors

Unfortunately, there is no truly universal HPLC detector with high sensitivity. Thus, there have been many attempts to find new principles that could lead to improved instrumentation. One interesting concept is the mass or evaporative detector, also known as the light scattering detector. The mobile phase is sprayed into a heated air stream, evaporating volatile solvents and leaving nonvolatile analytes as aerosols. These droplets or particles can be detected because they will scatter a beam of light (5). This method has been applied to the detection of fatty acids, lipids, and carbohydrates as an alternative to refractive index detection. For example, HPLC with light-scattering detection has been applied to the analysis of wheat flour lipids. In another recent study, monoacylglycerol was determined using HPLC with evaporative light-scattering detection and results that agreed with GC and SFC were obtained (2). Light scattering detectors are quite useful for the characterization of polymers by size-exclusion chromatography. Viscosity detectors are another specialized detector type, also utilized in conjunction with size-exclusion chromatography of polymers.

Radioactive detectors are widely used for pharmacokinetic and metabolism studies with radiolabeled drugs. Decay of a radioactive nucleus leads to excitation of a scintillator, which subsequently loses its excess energy by photon emission. Photons are counted by a photomultiplier tube and the number of counts per second is proportional to radiolabeled analyte (6).

Because the HPLC mobile phase often interferes with detection, various transport detectors also have been developed. The eluent is deposited onto a carrier (a wire belt or ceramic disc) that passes through an oven to evaporate solvent. Residual nonvolatile analytes are then detected via a flame ionization detector (see Chapter 33) or by pyrolysis to carbon dioxide.

These instruments have been used with lipids and carbohydrates, as alternatives to refractive index detection (5).

Recently, a novel chemiluminescent nitrogen detector (CLND) has been described. Nonnitrogenous compounds are transparent to this detector; thus, nitrogen-containing compounds can be detected without using chemical derivatization (section 32.2.4.7). Nitrogen-specific detection has been used to quantitate caffeine in coffee and soft drink beverages, and to analyze capsaicin in red hot peppers (2).

32.2.4.6 Coupled Analytical Techniques

To obtain more information about the analyte(s), eluent from an HPLC system can be passed on to a second analytical instrument, such as infrared (IR), nuclear magnetic resonance (NMR), or mass spectrometers (MS) (see Chapter 27, 30, and 29, respectively). This may be referred to as the use of a hyphenated technique. Unfortunately, the coupling of spectrometers with liquid chromatography (LC) has been slow to gain application due to many practical problems. In the case of HPLC with mass spectrometric detection (LC–MS), for example, as the liquid mobile phase evaporates, it swamps the vacuum in the mass spectrometer. This problem has been addressed by the development of commercial interfaces that allow solvent to be evaporated so that only analyte is carried to the spectrometer. The use of microbore HPLC columns with a low flow rate allows direct coupling of the two instruments (5, 10). Some applications of LC–MS are for the analysis of mycotoxins, nonvolatile pesticides, and emulsifying compounds.

32.2.4.7 Chemical Reactions

Detection sensitivity or specificity may sometimes be enhanced by converting the analyte to a chemical derivative with different spectral or redox characteristics. An appropriate reagent can be added to the sample prior to injection, i.e., pre-column derivatization, or combined with column effluent before it enters the detector, i.e., post-column derivatization. Automated amino acid analyzers utilize post-column derivatization, usually with ninhydrin, for reliable and reproducible analyses of amino acids. Pre-column derivatization of amino acids with o-phthalaldehyde or similar reagents permits highly sensitive HPLC determination of amino acids using fluorescence detection.

32.2.5 Recorder/Integrator/Data System

A detector provides an electronic signal related to the composition of the HPLC column effluent. It is the job

of the last element in the chain of HPLC instrumentation to display, and permit quantitation of, the peaks in the chromatogram. Until fairly recently, the strip chart recorder was the main output device, with retention times and peak height or area values being determined manually by the analyst, as discussed in Chapter 31. Today, these functions often are performed by an electronic integrator. Basically, this instrument monitors the HPLC detector signal and can, if correctly programmed by the analyst, recognize the start, maximum, and end of each chromatographic peak. These values then are used to determine retention times and peak areas. At the end of each run, a report is printed out that lists these data and post-run calculations, such as relative peak areas, areas as percentages of the total area, and relative retention times. If the system has been standardized, data from external or internal standards can be used to calculate analyte concentrations. Although the use of an electronic integrator can greatly simplify data reduction, it is important for the analyst to understand the capabilities and limitations of this instrument and subject its output to critical appraisal (5, 6).

An integrator may be a stand-alone device or microcomputer-based. Software packages to aid data evaluation are commercially available. For example, in pesticide residue analysis, analyte retention times can be calculated relative to an internal standard. The resulting relative retention times are compared with a data base for standards to determine probable analyte identity. In addition, computer-based integrators may be linked to central laboratory information management systems (LIMS) (see Chapter 6) as a way of facilitating the collection, analysis, and storage of data from several laboratory instruments.

32.3 SEPARATION MODES IN HPLC

The basic physicochemical principles underlying all liquid chromatographic separations—adsorption, partition, ion exchange, size exclusion, and affinity—are discussed in Chapter 31, and details will not be repeated here. The number of separation modes utilized in HPLC, however, is greater than that available to the classical chromatographer. This is attributable to the success of bonded phases, initially developed to facilitate classical liquid–liquid partition chromatography (Chapter 31, section 31.3.3.2.). In fact, reversed-phase HPLC is the most widely used separation mode in modern column liquid chromatography.

32.3.1 Normal Phase

32.3.1.1 Stationary and Mobile Phases

In normal-phase HPLC, the stationary phase is a polar adsorbant, such as bare silica or silica to which polar

nonionic functional groups—alcoholic hydroxyl, nitro, cyano (nitrile), or amino—have been chemically attached. Reactions similar to that described in section 32.2.3.2.2 are used, with terminal polar substituents being linked to the silica through a short hydrocarbon spacer (e.g., R_3 = —$CH_2CH_2CH_2NH_2$, aminopropyl) (13). These bonded phases are moderately polar and have advantages over bare silica: Solute–stationary phase interactions are less extreme and the surface is more uniform, resulting in better peak shapes.

The mobile phase for this mode consists of a nonpolar solvent, such as hexane, to which is added a more polar modifier, such as methylene chloride, to control solvent strength and selectivity. Solute retention, based on an adsorption/displacement process, can be modulated by varying the polarity or solvent strength of the mobile phase (Chapter 31, section 31.3.3.1). Solvent strength refers to the way a solvent affects the migration rate of the sample. Weak solvents increase retention (large k' values) and strong solvents decrease retention (small k' values). It is important to realize that the strength of a particular solvent depends solely on the chromatographic mode. For example, pentane is a weak solvent in normal-phase chromatography but a very strong solvent in the reversed-phase mode.

32.3.1.2 Applications of Normal-Phase HPLC

Normal-phase HPLC is best applied to the separation of compounds that are highly soluble in organic solvents, such as fat-soluble vitamins, or suffer from low stability in aqueous mobile phases, such as phospholipids. Compound classes (see Table 31-2), isomers, and highly hydrophilic species, such as carbohydrates (see Chapter 11), also may be resolved by normal-phase chromatography. Amino bonded-phase HPLC columns are utilized for the separation of carbohydrates (7).

32.3.2 Reversed Phase

32.3.2.1 Stationary and Mobile Phases

More than 70% of all HPLC separations are carried out in the reversed-phase mode which utilizes a nonpolar stationary phase and a polar mobile phase. The stationary phases most commonly employed are chemically bonded phases prepared by the reaction of silica surface silanols with an organochlorosilane as described in section 32.2.3.2.2. Usually, the R_3 group (Equation [1]) is an octadecyl (C_{18}) chain [—$(CH_2)_{17}$ CH_3]. Octadecylsilyl (ODS) bonded phases are one of the most popular reversed-phase packing materials. Shorter chain hydrocarbons, e.g., octyl (C_8) or butyl (C_4), or phenyl groups also can be attached to the silica surface. The use of monochlorosilanes leads to mono-

meric bonded phases, where a monomolecular organic layer is formed on the silica surface. Reacting silica with a di- or trichlorosilane leads to the formation of a polymeric layer. In the reaction of silica surface silanol groups with bulky organosilanes, only ca. 50% of the —OH moieties are derivatized. Since they are weakly acidic and can possess a negative charge, these residual unreacted silanols can contribute significantly to undesirable band broadening and tailing of some solutes, especially amines. For this reason, the silica is sometimes subjected to a second reaction (endcapping) with a small silylating reagent, such as trimethylchlorosilane.

Many silica-based reversed-phase columns are commercially available, and differences in their chromatographic behavior result from variation in the following (12):

1. Type of organic group bonded to the silica matrix, such as C_{18} versus phenyl
2. Chain length of organic moiety, such as C_8 versus C_{18}
3. Amount of organic moiety per unit volume of packing
4. Support particle size and shape
5. Matrix surface area and porosity
6. Bonded phase surface topology, such as monomeric versus polymeric
7. Concentration of free silanols

Polymeric packing materials eliminate the problem of residual silanols, provide increased pH stability, and offer additional selectivity parameters. Highly crosslinked poly(styrene-divinylbenzene) may be used directly in the reversed-phase mode or can be modified with various hydrophilic or hydrophobic functional groups, including C_{18}.

Reversed-phase HPLC utilizes polar mobile phases, usually water mixed with methanol, acetonitrile, or tetrahydrofuran. Solutes are retained due to hydrophobic interactions with the nonpolar stationary phase and are eluted in order of increasing hydrophobicity (decreasing polarity). Increasing the polar (aqueous) component of the mobile phase increases solute retention (larger k' values), whereas increasing the organic solvent content of the mobile phase decreases retention (smaller k' values). Various additives can serve additional functions. An amine, such as triethylamine, may be added to the mobile phase to deactivate residual silanols. Aqueous buffers may be employed to suppress or otherwise control the ionization of sample components. Although ionogenic compounds often can be resolved without them, ion-pair reagents may be used to facilitate chromatography of ionic species on reversed-phase columns. These reagents are ionic surfactants, such as octanesulfonic acid,

which can neutralize-charged solutes and make them more lipophilic. Depending upon the concentration of ion-pairing agent, retention can be varied continuously from a reversed-phase process to an ion-exchange process. Hence, ion-pair (paired-ion, ion-association) HPLC is sometimes included in discussions of ion-exchange chromatography or may even be treated as an independent separation mode.

32.3.2.2 Applications of Reversed-Phase HPLC

As previously stated, reversed-phase HPLC is widely used. Only a few important food-related applications are mentioned here. Reversed-phase has been the HPLC mode most used for analysis of plant proteins. Cereal proteins, among the most difficult of these proteins to isolate and characterize, are now routinely analyzed by this method (7). Both water- and fat-soluble vitamins (Chapter 18) can be analyzed by reversed-phase HPLC (2-4). The availability of fluorescence detectors has enabled researchers to quantitate very small amounts of the more than six possible forms of Vitamin B_6 (vitamers) in foods and biological samples. Figure 32-4 shows the separation of several of these vitamers in a rice bran extract (15). Ion-pair reversed-phase HPLC can be used to resolve carbohydrates on C_{18} bonded-phase columns (7). The constituents of soft drinks (caffeine, aspartame, etc.) can be rapidly separated using reversed-phase chromatography. Reversed-phase HPLC with a variety of detection methods, including RI, UV, light-scattering, and LC-MS, has been applied to the analysis of lipids (2-4, 7). Antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), can be extracted from dry foods and analyzed by reversed-phase HPLC with simultaneous UV and fluorescence detection (3). Food dyes, pigments (such as chlorophylls, carotenoids, and anthocyanins) (Chapter 19), and phenolic flavor compounds (such as vanillin), also are amenable to reversed-phase HPLC (2-4, 7).

32.3.3 Ion Exchange

32.3.3.1 Stationary and Mobile Phases

Packing materials for ion-exchange HPLC often are functionalized organic resins, namely sulfonated or aminated poly(styrene-divinylbenzene) (Chapter 31, section 31.3.3.3). Although microporous resins (of ≥8% crosslinking) can be used, macroporous (macroreticular) resins are much more satisfactory for HPLC columns due to their greater rigidity and permanent pore structure (section 32.2.3.2.3). Pellicular packings also are utilized, although a disadvantage is their limited ion-exchange capacity, i.e., the number of equiva-
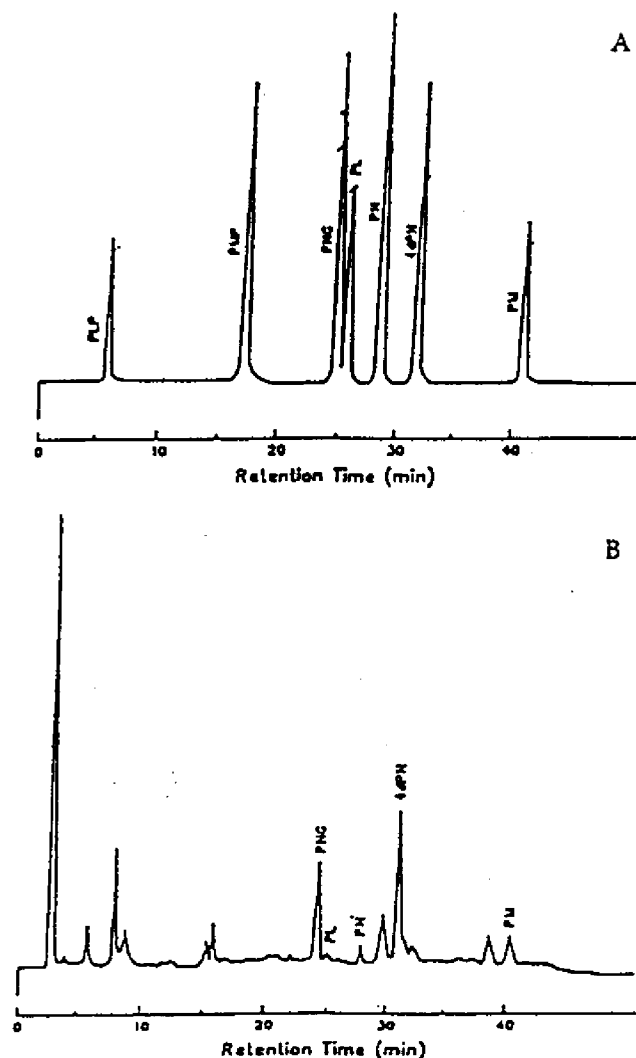
A

B

Retention Time (min)

lents of ion-exchange moieties per unit weight of packing material.

Silica-based bonded-phase ion exchangers also have been developed. Since ionogenic groups, provided by the chemically bonded layer, tend to be at the surface, ion exchange is rapid and good separation efficiency is obtained. As with other silica-based packings, however, mobile phase pH must be somewhat restricted.

The mobile phase in ion-exchange HPLC is usually an aqueous buffer, and solute retention is controlled by changing mobile phase ionic strength and/or pH. Gradient elution (gradually increasing ionic strength) is frequently employed. Ion-exchange is similar to an adsorption process, wherein mobile phase constituents compete with solutes for binding to sites on the stationary phase. Thus, increasing ionic strength of the mobile phase allows it to compete more effectively, and solute retention decreases. A change in mobile phase pH can affect the sample or the stationary phase or both. The functional groups of "strong" ion exchangers remain ionized across a broad pH range. Consequently, the binding capacity of these stationary phases is essentially constant, regardless of mobile phase pH, and changes in retention are due solely to alteration of solute charge. The ligand density or charge capacity of "weak" ion exchangers may be varied by manipulating mobile phase pH (Chapter 31, section 31.3.3.3).

32.3.3.2 Applications of Ion-Exchange HPLC

Ion-exchange HPLC has many applications, ranging from the detection of simple inorganic ions to analysis of carbohydrates and amino acids to the preparative purification of proteins.

32.3.3.2.1 Ion Chromatography Ion chromatography is simply high performance ion-exchange chromatography performed using a relatively low-capacity stationary phase (either anion- or cation-exchange) and, usually, a conductivity detector. All ions conduct an electric current; thus, measurement of electrical conductivity is an obvious way to detect ionic species. Because the mobile phase also contains ions, however, background conductivity can be relatively high. One step toward solving this problem is to use much lower capacity ion-exchange packing materials, so that more dilute eluents may be employed. In nonsuppressed or single-column ion chromatography, the detector cell is placed directly after the column outlet and eluents are carefully chosen to maximize changes in conductivity as sample components elute from the column. Suppressed ion chromatography utilizes an eluent that can be selectively removed. Originally, this was accomplished by the addition of a second suppressor column between the analytical column and the conductivity detector. Today, suppressor columns have been replaced by ion-exchange membranes (7). Suppressed ion chromatography permits the use of more concentrated mobile phases and gradient elution. Ion chromatography can be used to determine inorganic anions and cations, transition metals, organic acids, amines, phenols, surfactants, and sugars. Some specific examples of ion chromatography applied to food ma-

trices include the determination of organic and inorganic ions in milk; organic acids in coffee extract and wine; choline in infant formula; and trace metals, phosphates, and sulfites in foods. Figure 32-5 illustrates the simultaneous determination of organic acids and inorganic anions in coffee by ion chromatography.

32.3.3.2.2 Carbohydrates and Compounds of Biochemical Interest Both cation- and anion-exchange stationary phases are applied to HPLC of carbohydrates. Sugars and sugar derivatives can be separated on gel-type (microporous) polymeric cation-exchange resins in the H^+ form or loaded with Ca^{2+} or other metal counterions (Fig. 32-6). Various separation mechanisms are involved, depending on the counterion associated with the resin, degree of crosslinking (4 versus 8%), and type of carbohydrate (7). Chromatographic selectivity is mainly a function of the stationary phase. These columns are generally operated at elevated temperatures (ca. 85°C) to obtain the highest efficiency and resolution. It is essential to perform adequate sample cleanup and avoid undue pressure on these packing materials.

The advantage of separating carbohydrates by anion exchange is that retention and selectivity may be altered by changes in eluent composition. Anion-exchange HPLC with fluorescence detection has been used to quantitate oligosaccharides in commercial soybean lecithin. Charged oligosaccharides from hydrolyzed pectin-containing regions of apple were analyzed by anion-exchange HPLC in combination with thermospray mass spectrometry (2).

Anions in Coffee

1. Acetate
2. Glycolate
3. Quinate
4. Formate
5. Chloride
6. Tartrate
7. Oxalate
8. Fumarate
9. Phosphate
10. Citrate
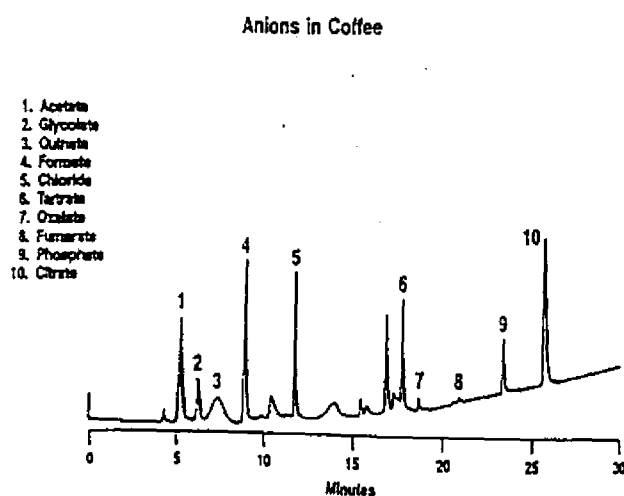
32-5 figure Ion-chromatographic analysis of organic acids and inorganic anions in coffee. Ten anions (listed) were resolved on an IonPac AS5A column (Dionex) using a sodium hydroxide gradient and suppressed conductivity detection. (Courtesy of Dionex Corp., Sunnyvale, CA.)
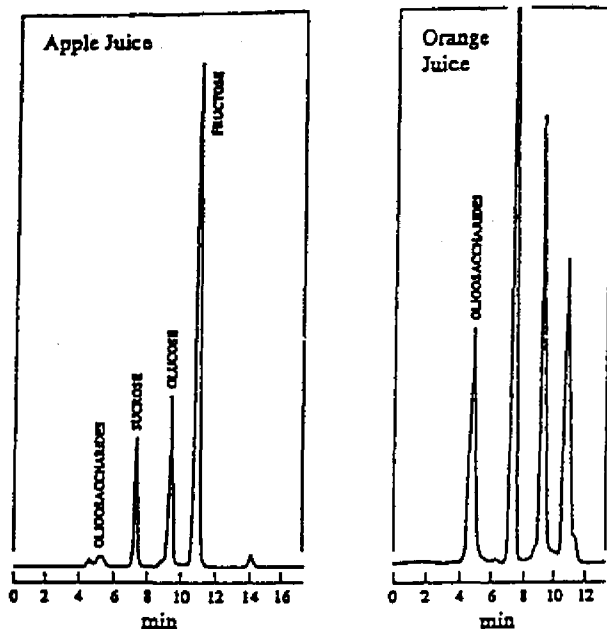
32-6 figure Cation-exchange separation of sugars in fruit juice. Calcium-form column (Interaction CHO-620; 30 × 0.65 cm; 90°C) eluted with distilled water at 0.5 ml/min. Refractive index detection. (Courtesy of Interaction Chromatography, San Jose, CA.)

Carbohydrate analysis has benefited greatly by the relatively recent introduction of a technique that involves anion-exchange HPLC at high pH ($\geq$12), and detection by a pulsed amperometric detector. Pellicular column packings (see section 32.2.3.2.2), consisting of nonporous latex beads coated with a thin film of strong anion-exchanger, provide the necessary fast exchange, high efficiency, and resistance to strong alkali.

Amino acids have been resolved on polymeric ion-exchangers for more than 30 years (see Chapter 16). Ion exchange is one of the most effective modes for HPLC of proteins and, recently, has been recognized as valuable for the fractionation of peptides.

32.3.4 Size Exclusion

Size-exclusion chromatography fractionates solutes solely on the basis of their size. Due to the limited separation volume available to this chromatographic mode, as explained in Chapter 31 (section 31.3.3.4), the peak capacity of a size-exclusion column is relatively small, i.e., 10–13 peaks (see Fig. 31-6). Thus, the "high performance" aspect of HPLC, which generally implies columns with 2000–20,000 theoretical plates, is not really applicable in the case of size exclusion. The main advantage gained from use of small particle packing materials is speed. Separation times of $\leq$60

min may be realized with 5–13 μm particles, compared to $\leq$24 hr separations using 30–150 μm particles in classical columns (7).

32.3.4.1 Column Packings and Mobile Phases

Size-exclusion packing materials or columns are selected so that matrix pore size matches the molecular weight range of the species to be resolved. Prepacked columns of microparticulate media are available in a wide variety of pore sizes. Hydrophilic packings, for use with water-soluble samples and aqueous mobile phases, may be surface-modified silica or methacrylate resins. Poly(styrene-divinylbenzene) resins are useful for nonaqueous size-exclusion chromatography of synthetic polymers.

The mobile phase in this mode is chosen for sample solubility, column compatability, and minimal solute–stationary phase interaction. Otherwise, it has little effect on the separation. Aqueous buffers are used for biopolymers, such as proteins and nucleic acids, both to preserve biological activity and to prevent adsorptive interactions. Tetrahydrofuran or dimethylformamide is generally used for size-exclusion chromatography of polymer samples, to ensure sample solubility.

32.3.4.2 Applications of High Performance Size-Exclusion Chromatography

Hydrophilic polymeric size-exclusion packings are used for the rapid determination of average molecular weights and degree of polydispersity of polysaccharides, including amylose, amylopectin, and other soluble gums such as pullulan, guar, and water-soluble cellulose derivatives. Molecular weight distribution can be determined directly from high performance size-exclusion chromatography, if low-angle laser light scattering (LALLS) is used for detection (7).

Size-exclusion HPLC has been shown to be a rapid, one-step method for assessing soybean cultivars on the basis of protein content. (Proteins in the extracts of nondefatted flours from five soybean cultivars were separated into six common peaks, and cultivars could be identified by the percent total area of the fifth peak.) Recently, a gel permeation liquid chromatographic method has been applied to the determination of polymerized triacylglycerols in oils and fats (2).

32.3.5 Affinity

Affinity chromatography is based on the principle that the molecules to be purified can form a selective but reversible interaction with another molecular species that has been immobilized on a chromatographic support. Columns packed with preactivated media on

which a ligand of interest can be immobilized in situ have greatly facilitated the use of high performance affinity chromatography. Although almost any material can be immobilized on a suitably activated support, the major ligands are proteins, nucleic acids, dyes, and lectins (Chapter 31, section 31.3.3.5). Affinity chromatography is used to purify many glycoproteins, as described in Chapter 16. An affinity technique that does not involve bioselective processes is metal chelate affinity chromatography. Ligands consist of immobilized iminodiacetic acid to which various metal ions, such as Cu^{2+} or Zn^{2+}, can be complexed. Coordination with these metal ions is the basis for separation of some proteins. Affinity chromatography using immobilized folate binding protein is an effective tool in purifying sample extracts for HPLC analysis of folates in foods and biological materials [for example, reference (16)].

32.4 DEVELOPING A SEPARATION

There may be numerous ways to accomplish a chromatographic separation for a particular compound. In many cases, the analyst will follow a standard laboratory procedure or published methods. In the case of a sample that has not been previously analyzed, some useful guidelines for method development are presented in references (1), (6), and (17).

Begin by evaluating what is known about the sample and define the goals of the separation. How many components need to be resolved? What degree of resolution is needed? Is qualitative or quantitative information needed? Molecular weight (or molecular weight range), polarity, and ionic character of the sample will guide the choice of separation mode. Figure 32-7 shows that more than one correct choice may be possible. (For example, small ionic compounds may be separated by ion-exchange, ion-pair reversed-phase, or reversed-phase HPLC.) In this case, the analyst's choice may be based on convenience, experience, and personal preference.

Having chosen a separation mode for the sample at hand, one must select an appropriate column, elution conditions, and a detection method. Trial experimental conditions may be based on the results of a literature search, the analyst's previous experience with similar samples, or general recommendations from chromatography experts [see, for example, reference (17)].

To achieve separation of sample components by all modes except SEC, one may utilize either isocratic or gradient elution. Isocratic elution is the most simple and widely used technique, in which solvent composition and flow rate are held constant. Gradient elution involves reproducibly varying mobile phase composition or flow rate (flow programming) during an HPLC

analysis. Gradient elution is used when sample components possess a wide range of polarities, so that an isocratic mobile phase does not elute all components within a reasonable time. Increasing the "strength" of the mobile phase (section 32.3.1.1), either gradually or in a stepwise fashion, shortens the analysis time. (Gradient elution is routinely used for HPLC of large molecules.)

Although method development may begin with an isocratic mobile phase, possibly of intermediate solvent strength, Snyder (17) recommends using gradient elution for the initial separation. This ensures that some level of separation will be achieved within a reasonable time period and nothing is likely to remain on the column. Data from this initial "scouting" run allows one to determine if isocratic or gradient elution is needed, and to estimate optimal isocratic mobile phase composition or gradient range. (The use of a gradient scouting run does *not* presuppose that the final method will use gradient elution.)

Once an initial separation has been achieved, the analyst can proceed to optimize resolution. This generally involves manipulation of mobile phase variables, including the nature and percentage of organic components, pH, ionic strength, nature and concentration of additives (such as ion-pairing agents), and temperature. In the case of gradient elution, gradient steepness (slope) is another variable to be optimized.

32.5 SAMPLE PREPARATION AND DATA EVALUATION

It is important to recognize that the success or failure of HPLC methods, as with applications of other analytical techniques, is ultimately dependent upon the adequacy of sample preparation. Other methodologies, some of which are listed in Table 32-1, generally are needed to remove interfering materials prior to chromatography. Variables such as extraction efficiency, analyte stability, and consistency of chemical or enzymatic pretreatment must be considered during each sample preparation step. This is especially critical when microconstituents, such as Vitamin B_6 compounds (15) or folates (16), are to be analyzed. The use of HPLC to analyze foods for pesticide, mycotoxin, and drug residues also requires fastidious sample preparation and data evaluation. In the future, sample preparation may become much more automated, with robots being used to perform repetitive procedures. Already, they can carry out extractions and derivatizations, prepare solutions, use solid-phase cartridges, evaporate, and centrifuge (5).

The data acquired from an HPLC analysis must be evaluated from several aspects. Identification and quantitation of chromatographic peaks are discussed in Chapter 31 (sections 31.3.4.3.2 and 31.3.4.3.4). As
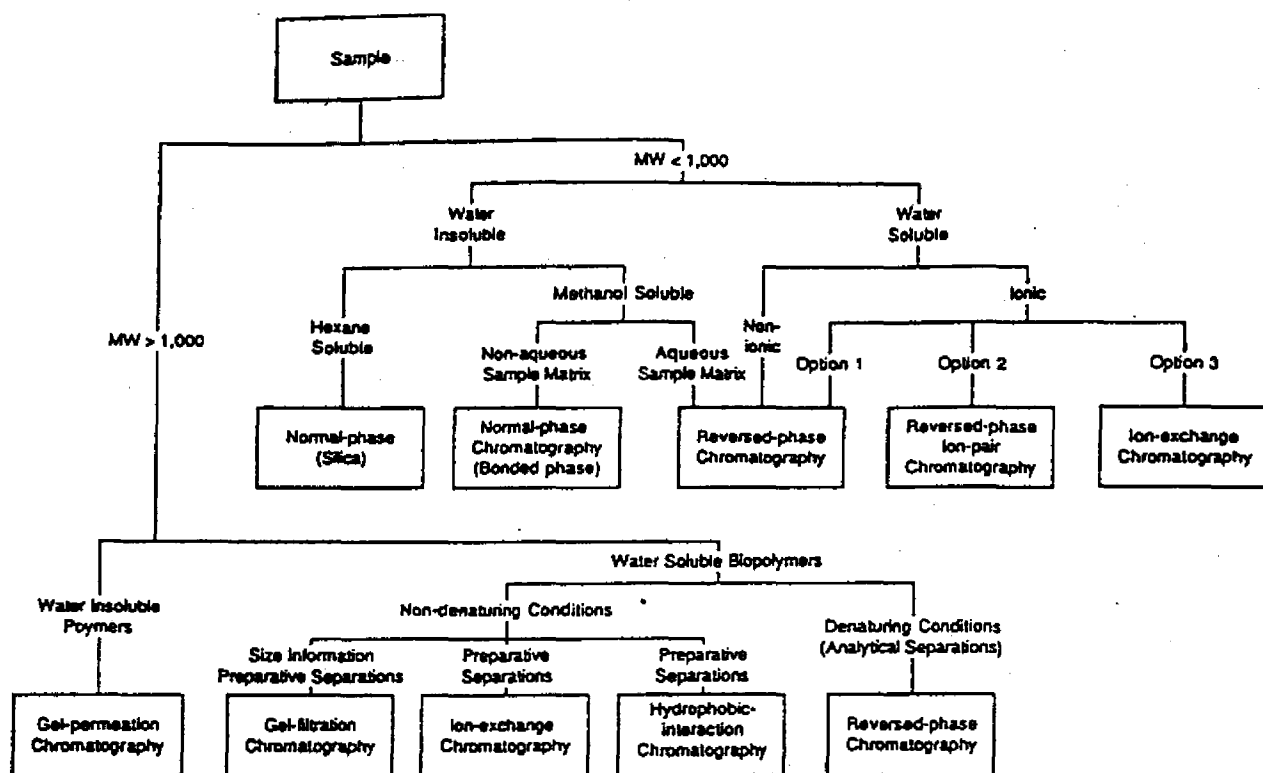
32-7 figure A schematic diagram for choosing a chromatographic separation mode based on sample molecular weight and solubility. [From (6), used with permission.]

32-1 table Sample Preparation Steps Prior to HPLC

Liquid handling	Centrifugation
Solvent extraction	Evaporation
pH change	Freeze drying
Chemical derivatization	Enzyme digestion
Solid phase extraction	Hydrolysis
Soxhlet extraction	Column switching
Steam distillation	Low-temperature storage
Supercritical fluid extraction	Filtration
Homogenization	Column chromatography
Precipitation	Microwave irradiation
Dialysis	

From (6), used with permission.

stated previously, the fact that the retention time of an unknown and a standard are equivalent does not prove that the two compounds are identical. Other techniques are needed to confirm peak identity. For example:

1. A sample can be spiked with a small amount of added standard; only the height of the peak of interest should increase, with no change in retention time, peak width, or shape.

2. A diode array detector can provide absorption spectra of designated peaks. Although identical spectra do not prove identity, a spectral difference confirms that sample and standard peaks are different compounds.

3. In the absence of spectral scanning capability, other detectors, such as absorption or fluorescence, may be used in a ratioing procedure. Chromatograms of sample and standard are monitored at each of two different wavelengths. The ratio of peak areas at these wavelengths should be the same if sample and standard are identical.

4. Peaks of interest can be collected and subjected to additional chromatographic (different separation mode) or nonchromatographic (e.g., spectroscopic) analysis.

The quantitative validity of an HPLC analysis also must be confirmed. Standards should be included during each analytical session, since detector response may vary from day to day. Analyte recovery should be checked periodically. This involves addition of a

known quantity of standard to a sample (usually before extraction) and determination of how much is recovered during subsequent analysis. During routine analyses, it is highly desirable to include a control or check sample, a material of known composition. This material is analyzed parallel to unknown samples. When the concentration of analyte measured in the control falls outside an acceptible range, data from other samples analyzed during the same period should be considered suspect. Carefully analyzed food samples and other substances are available from the National Institute of Standards and Technology (formerly the National Bureau of Standards) for use in this manner.

32.6 SUMMARY

High performance liquid chromatography is a chromatographic technique of great versatility and analytical power. A basic HPLC system consists of a pump, injector, column(s), detector, and recorder/integrater/data system. The pump delivers mobile phase through the system. An injector allows sample to be placed into the flowing mobile phase for introduction onto the column. The HPLC column, connected between injector and detector, consists of stainless steel hardware filled with a packing material. Various auxilliary columns may be used prior to the analytical column. Detectors used in HPLC include UV-Vis absorption, fluorescence, refractive index, electrochemical, and several other types, including coupled analytical systems. Detection sensitivity or specificity sometimes can be enhanced by chemical derivatization of the analyte. While a strip-chart recorder serves to record the basic results of a chromatographic separation, electronic integrators and computer-controlled systems offer additional data-handling capability. A broad variety of column packing materials has contributed greatly to the widespread use of HPLC. These column packing materials may be categorized as silica-based (porous silica, bonded phases, pellicular packings) or polymeric (microporous, macroporous). The success of silica-based bonded phases has expanded the applications of normal-phase and reversed-phase modes of separation in HPLC. Separation also can be achieved by utilizing the principles of ion-exchange, size-exclusion, and affinity chromatography. HPLC is widely used for the analysis of small molecules and ions, such as sugars, vitamins, and amino acids, and is applied to the separation and purification of macromolecules, such as proteins, nucleic acids, and polysaccharides. As with other analytical techniques, careful sample preparation and confirming the identity of resolved species are essential to the success of HPLC methods.

32.7 STUDY QUESTIONS

1. Why might you choose to use HPLC rather than traditional low-pressure column chromatography?
2. What is a guard column and why is it used?
3. Give three general requirements for HPLC column packing materials. Describe and distinguish among porous silica, bonded phases, pellicular, and polymeric column packings, including the advantages and disadvantages of each type.
4. What is the primary function of an HPLC detector (regardless of type)? What factors would you consider in choosing an HPLC detector? Describe three different types of detectors and explain the principles of operation for each.
5. A sample containing compounds A,B, and C is analyzed via HPLC using a column packed with a silica-based C_{18} bonded phase. A 1:5 solution of ethanol and H_2O was used as the mobile phase. A UV detector was used, and the following chromatogram was obtained.
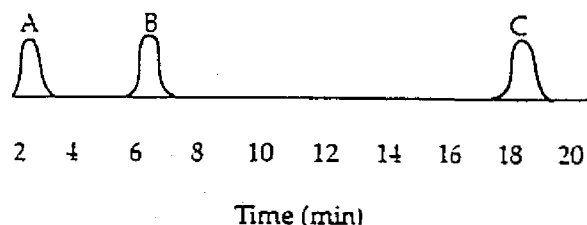
Assuming that the separation of compounds is based on their polarity,
a. Is this normal- or reversed-phase chromatography? Explain your answer.
b. Which compound is the most polar?
c. How would you change the mobile phase so compound C would elute sooner, without changing the relative positions of compounds A and B? Explain why this would work.

6. Ion chromatography has recently become a widely promoted chromatographic technique in food analysis. Describe ion chromatography and give at least two examples of its use.
7. Describe one application each for ion-exchange and size-exclusion HPLC.

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33

chapter

Gas Chromatography

Gary A. Reineccius

33.1 INTRODUCTION

The first publication on gas chromatography (GC) was in 1952 (1), while the first commercial instruments were manufactured in 1956. James and Martin (1) separated fatty acids by GC, collected the column effluent, and titrated the individual fatty acids for quantitation. GC has advanced greatly since that early work and is now considered to be a mature field that is approaching theoretical limitations.

The types of analysis that can be done by GC are very broad. GC has been used for the determination of fatty acids, triglycerides, cholesterol and other sterols, gases, solvent analysis, water, alcohols, and simple sugars, as well as oligosaccharides, amino acids and peptides, vitamins, pesticides, herbicides, food additives, antioxidants, nitrosamines, polychlorinated biphenyls (PCBs), drugs, flavor compounds, and many more. The fact that GC has been used for these various applications does not necessarily mean that it is the best method—often better choices exist. GC is ideally suited to the analysis of thermally stable volatile substances. Substances that do not meet these requirements (e.g., sugars, oligosaccharides, amino acids, peptides, and vitamins) are more suited to analysis by a technique such as high performance liquid chromatography (HPLC) or supercritical-fluid chromatography (SFC). Yet gas chromatographic methods appear in the literature for these substances.

This chapter will discuss sample preparation for GC, gas chromatographic hardware, columns, and chromatographic theory as it uniquely applies to gas chromatography. Texts devoted to GC in general (2–4) and food applications in particular (5) should be consulted for more detail.

33.2 SAMPLE PREPARATION FOR GAS CHROMATOGRAPHY (GC)

33.2.1 Introduction

One cannot generally directly inject a food product into a GC without some sample preparation. The high temperatures of the injection port will result in the degradation of nonvolatile constituents and create a number of false GC peaks corresponding to the volatile degradation products formed. In addition, very often the constituent of interest must be isolated from the food matrix simply to permit concentration such that it is at detectable limits for the GC or isolate it from the bulk of the food. Thus, one must generally do some type of sample preparation, component isolation, and concentration prior to GC analysis.

Sample preparation often involves grinding, homogenization, or otherwise reducing particle size. There is substantial documentation in the literature showing that foods may undergo changes during sample storage and preparation. Many foods contain active enzyme systems that will alter the composition of the food product. This is very evident in the area of flavor work (6–8). Inactivation of enzyme systems via high temperature–short time thermal processing, sample storage under frozen conditions, drying the sample, or homogenization with alcohol may be necessary (see Chapter 5).

Microbial growth or chemical reactions may also occur in the food during sample preparation. Chemical reactions will often result in the formation of volatiles that will again give false peaks on the GC. Thus, the sample must be maintained under conditions such that degradation does not occur. Microorganisms are often inhibited by chemical means (e.g., sodium fluoride), thermal processing, drying, or frozen storage.

33.2.2 Isolation of Solutes From Foods

The isolation procedure may be quite complicated depending upon the constituent to be analyzed. For example, if one were to analyze the triglyceride bound fatty acids in a food, one would first have to extract the lipids (free fatty acids; mono; di; and triglycerides; sterols; fat-soluble vitamins, etc.) from the food (e.g., by solvent extraction), and then isolate only the triglyceride fraction (e.g., by adsorption chromatography on silica). The isolated triglycerides then would have to be treated to first hydrolyze the fatty acids from the triglycerides and subsequently to form esters to improve gas chromatographic properties. The two latter steps might be accomplished in one reaction by trans-esterification (e.g., borontriflouride in methanol) as described in Chapter 14, section 14.6.1. Thus many steps involving several types of chromatography may be used in sample preparation for GC analysis.

The analysis of volatiles in foods (e.g., packaging or environmental contaminants, alcohols and flavors or off-flavors) may be a simpler task. These materials for GC analysis may be isolated by headspace analysis (static or dynamic), distillation, preparative chromatography (e.g., solid-phase extraction, column chromatography on silica gel), simple solvent extraction, or some combination of these basic methods. The procedure used will depend on the food matrix as well as the compounds to be analyzed. The primary considerations are to isolate the compounds of interest from non-volatile food constituents (e.g., carbohydrates, proteins, vitamins) or those that would interfere with GC (e.g., lipids). Some of the chromatographic methods that might be applied to this task have been discussed in the basic chromatography chapter (Chapter 31) of this text. Methods for the isolation of volatile substances (e.g., headspace analysis, distillation, and extraction methods) have not been discussed in this

text, so they will be covered briefly as they pertain to the isolation of components for gas chromatographic analysis.

It should be emphasized that the isolation procedure used is critical in determining the results obtained. An improper choice of method or poor technique at this step negates the best gas chromatographic analysis of the isolated solutes. The influence of isolation technique on gas chromatographic analysis of a mixture of aroma compounds ranging from ethanol to isoeugenol is evident from Fig. 33-1. All of the chromatograms (presented as bar charts) shown in this figure were obtained from the analysis of the *same aqueous model system*. The gross differences in GC profile are simply due to the biases introduced by the selectivity of the isolation method. These biases are discussed in the sections that follow.

33.2.2.1 Headspace Methods

One of the simplest methods of isolating volatile compounds from foods is by direct injection of the headspace vapors above a food product. Unfortunately, this method does not provide the sensitivity needed for trace analysis (Fig. 33-1). Instrumental constraints typically limit headspace injection volumes to 5 ml or less. Therefore, only volatiles present in the headspace at concentrations greater than 10^{-7} g/liter headspace would be at detectable levels (using a flame ionization detector).

Direct headspace sampling has been used extensively where rapid analysis is necessary and major component analysis is satisfactory. Examples of method applications include measurement of hexanal as an indicator of oxidation (10, 11) and 2-methyl propanal, 2-methyl butanal, and 3-methyl butanal as indicators of nonenzymatic browning (12). The determination of residual solvents in packaging materials also may be approached by this method.

Headspace concentration techniques (often called dynamic headspace or purge and trap methods) have found wide usage in recent years. This concentration method may involve simply passing large volumes of headspace vapors through a cryogenic trap or, alternatively, a more complicated extraction and/or adsorption trap. A simple cryogenic trap offers some advantages and disadvantages. A cryo trap (if properly designed and operated) will collect headspace vapors irrespective of compound polarity and boiling point. However, water is typically the most abundant volatile in a food product, and, therefore, one collects an aqueous distillate of the product aroma. This distillate must be extracted with an organic solvent, dried, and then concentrated for analysis. These additional steps add analysis time and provide opportunity for sample con-

tamination. Recently, the use of adsorbent traps has become the most common means to concentrate headspace vapors.

Adsorbent traps offer the advantages of providing a water-free volatile isolate (trap material typically has little affinity for water) and are readily automated. The adsorbent initially used for headspace trapping was charcoal. The charcoal was either solvent extracted (CS), or thermally desorbed with backflushing (inert gas) to recover the adsorbed volatiles. The use of synthetic porous polymers as headspace trap material now dominates. Initially, Tenax (a porous polymer very similar to the skeleton of ion-exchange resins) was most commonly used; however, combinations of Tenax and other polymers are now seeing greater application. These polymers exhibit good thermal stability and reasonable capacity. Adsorbent traps are generally placed in a closed system and loaded, desorbed, and so on via the use of automated multiport valving systems.

The automated closed system approach provides reproducible GC retention times and quantitative precision necessary for some studies. The primary disadvantage of adsorbent traps is their differential adsorption affinity and limited capacity. Buckholz et al. (13) have shown that the most volatile peanut aroma constituents will break through two Tenax traps in series after purging at 40 ml/min for only 15 min. Therefore, the GC profile may only poorly represent the actual food composition due to biases introduced by the purging and trapping steps (Fig. 33-1).

33.2.2.2 Distillation Methods

Distillation processes are quite effective at isolating volatile compounds from foods for GC analysis (Fig. 30-1, graph labeled Atmospheric SDE). Product moisture or outside steam is used to heat and codistill the volatiles from a food product. This means that a very dilute aqueous solution of volatiles results, and a solvent extraction must be performed on the distillate to permit concentration for analysis. The distillation method most commonly used today is some modification of the original Nickerson–Likens distillation head. In this apparatus, a sample is boiled in one side flask and an extracting solvent in another. The product steam and solvent vapors are intermixed and condensed; the solvent extracts the organic volatiles from the condensed steam. The solvent and extracted distillate return to their respective flasks and are distilled to again extract the volatiles from the food. While this method is convenient and efficient, artifacts from solvents used in extraction, antifoam agents, steam supply (contaminated water), thermally induced chemical changes, and leakage of contaminated laboratory air into the system may contaminate the volatile isolate.

PURGE AND TRAP

SPLITLESS HEADSPACE

BATCH EXTRACTION
Dichloromethane

ATMOSPHERIC SDE
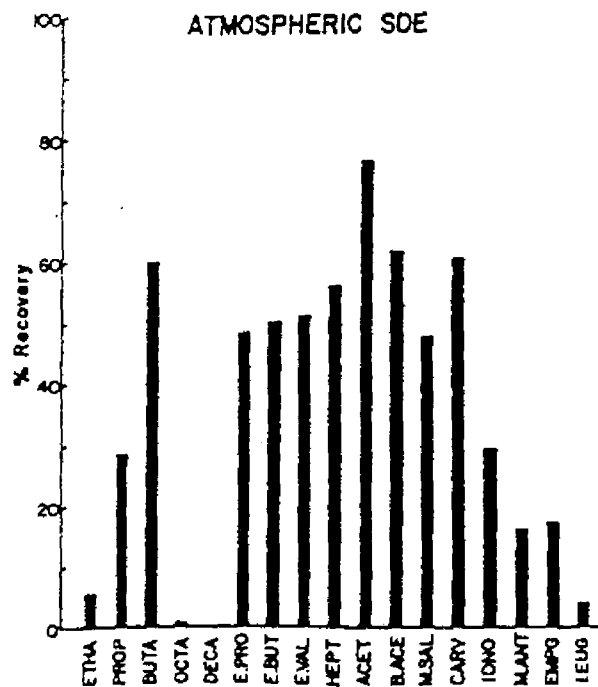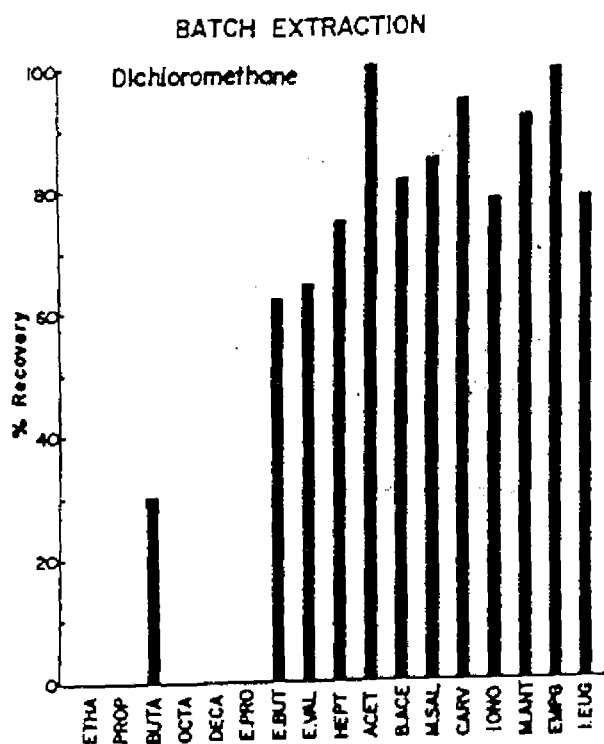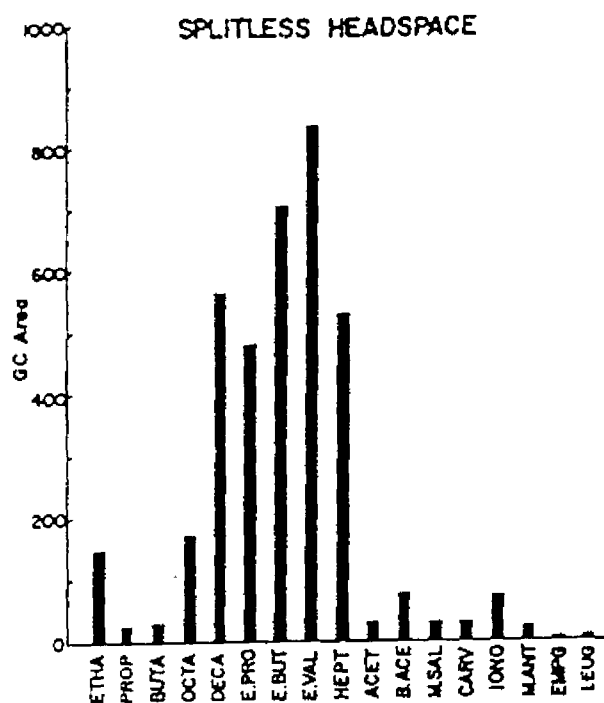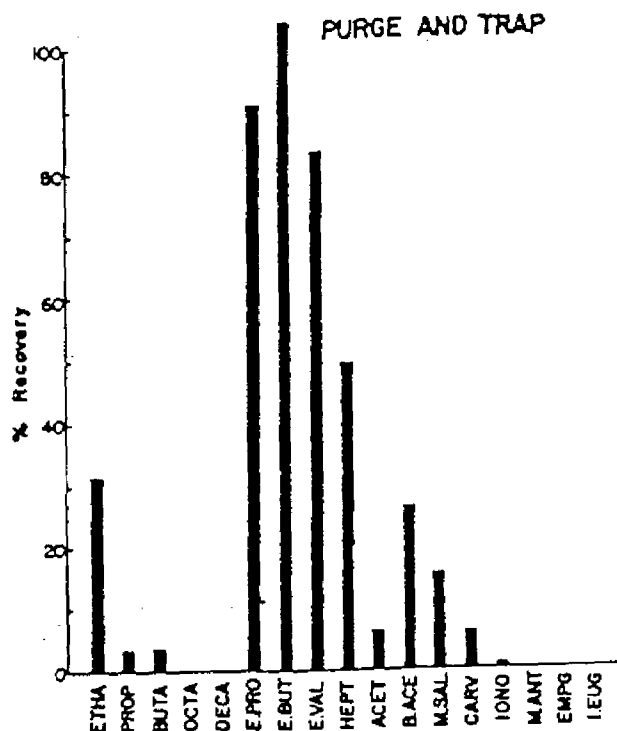
33-1 figure

Comparison of methods for the isolation of volatiles from aqueous model systems. (All bars should be equal height if equally recovered). [Adapted from (9) with permission.]

33.2.2.3 Solvent Extraction

Solvent extraction is often the preferred method for the recovery of volatiles from foods (Fig. 33-1). Recovery of volatiles will depend upon solvent choice and the solubility of the solutes being extracted. Solvent extraction typically involves the use of an organic solvent (unless sugars, amino acids, or some other water-soluble components are of interest). Extraction with organic solvents limits the method to the isolation of volatiles from fat-free foods (e.g., wines, some breads, fruit and berries, some vegetables, and alcoholic beverages), or an additional procedure must be employed to separate the extracted fat from the isolated volatiles (e.g., a chromatographic method). Fat will otherwise interfere with subsequent concentration and GC analysis.

Solvent extractions may be carried out in quite elaborate equipment, such as supercritical CO_2 extractors, or can be as simple as a batch process in a separatory funnel. Batch extractions can be quite efficient if multiple extractions and extensive shaking are used (14). The continuous extractors (liquid-liquid) are more efficient but require more costly and elaborate equipment.

33.2.2.4 Solid-Phase Extraction

The extractions discussed above involved the use of two immiscible phases (water and an organic solvent). However, a newer and very rapidly growing alternative to such extractions is solid-phase extraction (15, 16). In this technique, a liquid sample (most often aqueous based) is passed through a column (2-10 ml volume) filled with chromatographic packing or a Teflon® filter disk that has the chromatographic packing imbedded in it. The chromatographic packing may be any of a number of different materials (e.g., ion-exchange resins or a host of different reversed-or normal-phase HPLC column packings).

When a sample is passed through the cartridge or filter, solutes that have an affinity for the chromatographic phase will be retained on the phase while those with little or no affinity will pass through. The phase is next rinsed with water, perhaps a weak solvent (e.g., pentane) if a reversed-phase technique is used, and then a stronger solvent (e.g., dichloromethane). The strong eluant is chosen such that it will remove the solutes of interest.

Solid-phase extraction has numerous advantages over traditional liquid-liquid extractions including: (1) Less solvent is required; (2) it is faster; (3) less glassware is needed (less cost and potential for contamination); (4) better precision and accuracy; (5) minimal solvent evaporation for further analysis (e.g. gas chromatography); and (6) it is readily automated.

The most recent version of this method is called

solid phase microextraction (SPME, 17). In this adaptation, the phase is bound onto a fine fused silica filament (ca. the size of a 10-µl syringe needle). The filament can be immersed in a sample or in the headspace above a sample. After the desired loading time, the filament is pulled into a protective metal sheath, removed from the sample, and it is forced through the septum of a gas chromatograph where the adsorbed volatiles are thermally desorbed from the filament. For SPME, one can readily see the advantages of simplicity and lack of solvent usage. The primary concerns are for sensitivity limitations, precision, and life of the filament. If the filament must be replaced (breakage), there is the issue of reproducibility of the new versus old filament.

33.2.2.5 Direct Injection

It is theoretically possible to analyze some foods by direct injection of the food into a gas chromatograph. Assuming one can inject a 2- to 3-µL sample into a GC and the GC has a detection limit of 0.1 ng (0.1 ng/2 µl), one could detect volatiles in the sample at concentrations greater than 50 ppb. Problems with direct injection arise due to thermal degradation of any nonvolatile food constituents, damage to the GC column, decreased separation efficiency due to water in the food sample, contamination of the column and injection port by nonvolatile materials, and reduced column efficiency due to slow vaporization of volatiles from the food (injection port temperatures are reduced to minimize thermal degradation of the nonvolatile food constituents). Despite these concerns, direct injection is commonly used to determine oxidation in vegetable oils (18, 19). A relatively large volume of oil (50-100 µl) can be directly injected into an injection port of a GC that has been packed with glass wool. Since vegetable oils are reasonably thermally stable and free of water, this method is particularly well suited to oil analysis.

There are numerous other approaches for the isolation of volatiles from foods. Some are simple variations of these methods, while others are unique. Several review articles are available that provide a more complete view of methodology (20-22).

33.2.3 Sample Derivatization

The compounds one wishes to determine by GC must be thermally stable under the GC conditions employed. Thus, for some compounds (e.g., pesticides, aroma compounds, PCBs, and volatile contaminants) the analyst can simply isolate the components of interest from a food as discussed above and directly inject them into the GC. For compounds that are thermally unstable, too low in volatility (e.g., sugars and amino acids), or yield poor chromatographic separation due

to polarity (e.g., phenols or acids), a derivatization step must be included prior to GC analysis (see also Chapters 10 and 13). A listing of some of the reagents used in preparing volatile derivatives for GC is given in Table 33-1. The conditions of use for these reagents are often specified by the supplier or can be found in the literature.

33.3 GAS CHROMATOGRAPHIC HARDWARE AND COLUMNS

The major parts of a GC are the gas supply and regulators, injection port, oven, column, detector, electronics, and recorder/data handling system (Fig. 33-2). The hardware as well as operating parameters used in any GC analysis must be accurately and completely recorded. The information that must be included is presented in Table 33-2.

33.3.1 Gas Supply System

The gas chromatograph will require at least a supply of carrier gas, and most likely, gases for the detector (e.g., hydrogen and air for a flame ionization detector). The gases used must be of high purity and all regulators, gas lines, and fittings of good quality. The regulators should have stainless steel rather than polymer diaphragms since polymers will give off volatiles that may contribute peaks to the analytical run. All gas lines must be clean and contain no residual drawing oil. The

gas lines should have traps in line to remove any moisture and contaminants from the incoming gases. These traps must be periodically replaced to maintain effectiveness.

33.3.2 Injection Port

33.3.2.1 Hardware

The injection port serves the purpose of providing a place for sample introduction, its vaporization, and possibly some dilution and splitting. Liquid samples make up the bulk of materials analyzed by GC, and they are always done by syringe injection (manual or automated). The injection port contains a soft septum that provides a gas-tight seal but can be penetrated by a syringe needle for sample introduction.

The sample must be vaporized in the injection port in order to pass through the column for separation. This vaporization can occur quickly by flash evaporation (standard injection ports) or slowly in a more gentle manner (temperature programmed injection port or on-column injection). The choice depends upon the thermal stability of the analytes.

The injection port may serve the additional function of splitting the injection so that only a portion of the analyte goes on the column. Capillary columns have limited capacity, and the injection volume may have to be reduced to permit efficient chromatography.

Due to the various sample as well as instrumental

33-1
table

Reagents Used for Making Volatile Derivatives of Food Components for GC Analysis

Reagent	Chemical Group	Food Constituent
Silyl reagents Trimethylchlorosilane/hexamethyldisilazane BSA [N,O-bis(trimethylsilyl) acetamide] BSTFA [N,O-bis (trimethylsilyl) trifluoroacetamide] t-BuDMCS (t-butyldimethyl-chlorosilyl/imidazole)	Hydroxy, amino carboxylic acids	Sugars, sterols, amino acids
Esterifying reagents Methanolic HCl Methanolic sodium methoxide N,N-Dimethylformamide dimethyl acetal Boron trifluoride (or trichloride)/methanol	Carboxylic acids	Fatty acids, amines, amino acids, triglycerides, wax esters, phospholipids, cholesteryl esters
Miscellaneous Acetic anhydride/pyridine	Alcoholic and phenolic	Phenols, aromatic hydroxyl groups, alcohols
N-trifluoroacetylimidazole & N-heptafluorobutyrlimidizole	Hydroxy and amines	Same as above
Alkylboronic acids	Polar groups on neighboring atoms	
O-alkylhydroxylamine	Compounds containing both hydroxyl and carbonyl groups	Ketosteroids, prostaglandins
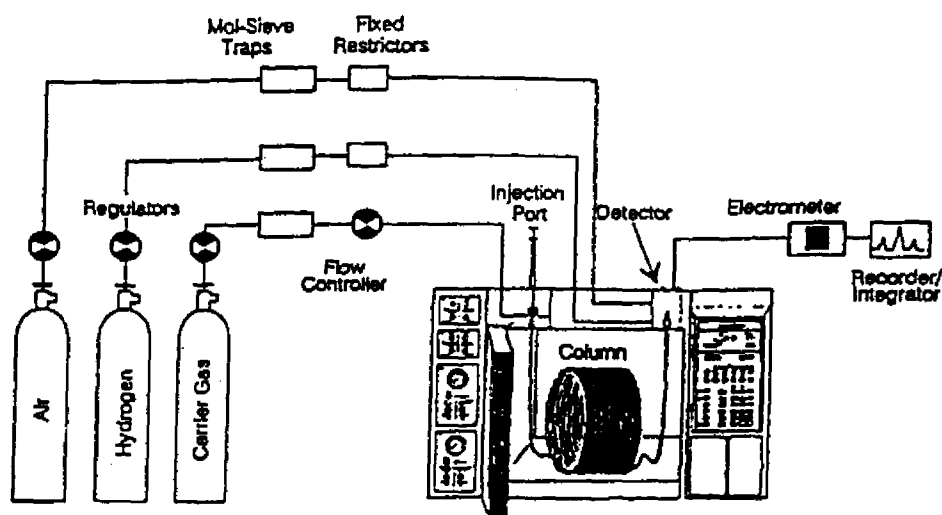
33-2 figure Diagram of a gas chromatographic system. (Courtesy of Hewlett Packard Co., Analytical Customer Training, Atlanta, GA.)

33-2 table Gas Chromatographic Hardware and Operating Conditions to be Recorded for All GC Separations

Parameter	Description
Sample	Name and injection volume
Injection	Type of injection (e.g., split versus splitless and conditions (injection port flow rates)
Column	Length, diameter (material-packed columns), and manufacturer
Packing/phase	Packed columns—solid support; size mesh; coating; loading (%)
	Capillary columns—phase material and thickness
Temperatures	Injector; detector; oven and any programming information
Carrier gas	Flow rate (velocity) and type
Detector	Type
Data output	Attenuation and chart speed

requirements, there are several different designs of injection ports available. These include the standard heated port (split or splitless, Fig. 33-3), temperature programmed, and on-column injectors. The standard injection port is operated about 20°C warmer than the maximum column oven temperature. The sample may be diluted with carrier gas to accomplish a split (1:50–1:100 preferred), whereby only a small portion of the analyte goes on the column or a mode whereby all of the analyte goes on the column (spitless injection). Splitless injection requires the use of a sample solvent that has a boiling point about 20°C above the initial column temperature. The solvent must recondense in the

column for acceptable chromatography of early eluting compounds.

For temperature-programmed injection ports, the sample is introduced into an ambient temperature port and then it is temperature programmed to some desired temperature. On-column is a technique whereby the sample is actually introduced into the column whose temperature is at that of the GC oven or that of the room. Either technique is desired for temperature sensitive analytes.

33.3.2.2 Sample Injection

Samples may be introduced into the injection port using a manual syringe technique or an automated sampling system. Manual sample injection is generally the largest single source of poor precision in GC analysis. Ten-microliter syringes are usually chosen since they are more durable than the microsyringes, and sample injection volumes typically range from 1 to 3 µl. These syringes will hold about 0.6 µl in the needle and barrel (this is in addition to that measured on the barrel). Thus the amount of sample that is injected into the GC depends upon the proportion of this 0.6 µl that is included in the injection and the ability of the analyst to accurately read the desired sample volume on the syringe barrel. This can be quite variable for the same analyst and be grossly different between analysts.

33.3.3 Oven

The oven controls the temperature of the column. In GC, one takes advantage of both an interaction of the
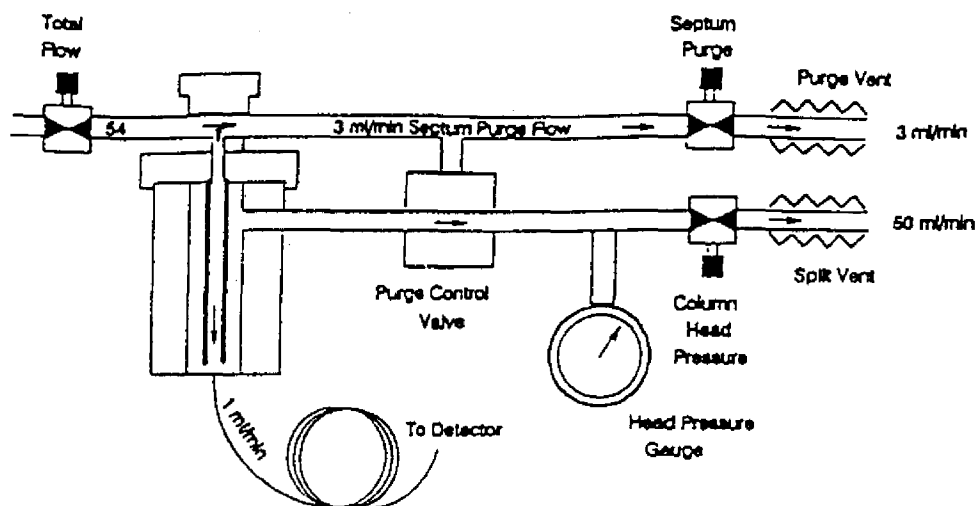
Schematic of a GC injection port. (Courtesy of Hewlett-Packard Co., Analytical Customer Training, Atlanta, GA.)

analyte with the stationary phase and the boiling point for separation of compounds. Thus, the injection is often made at a lower oven temperature and is then temperature programmed to some elevated temperature. While analyses may be done isothermally, compound elution time and resolution are extremely dependent upon temperature, so temperature programmed runs are most common. It should be obvious that higher temperatures will cause the sample to elute faster and, therefore be at a cost of resolution. Oven temperature program rates can range from as little as 0.1°C/min to the maximum temperature heating rate that the GC can provide. A rate of 2–10°C/min is most common.

33.3.4 Column and Stationary Phases

The GC column may be either packed or capillary. Early chromatography was done on packed columns, but the advantages of capillary chromatography so greatly outweigh those of packed column chromatography that few packed column instruments are sold any longer (Fig. 33-4). While some use HRGC (high resolution gas chromatography) to designate capillary GC, GC today means capillary chromatography to most individuals.

33.3.4.1 Packed Columns

The packed column is most commonly made of stainless steel or glass and may range from 1.6 to 12.7 mm in outer diameter and be 0.5–5.0 m long (generally 2–3 m). It is packed with a granular material consisting of a "liquid" coated on an allegedly inert solid support.
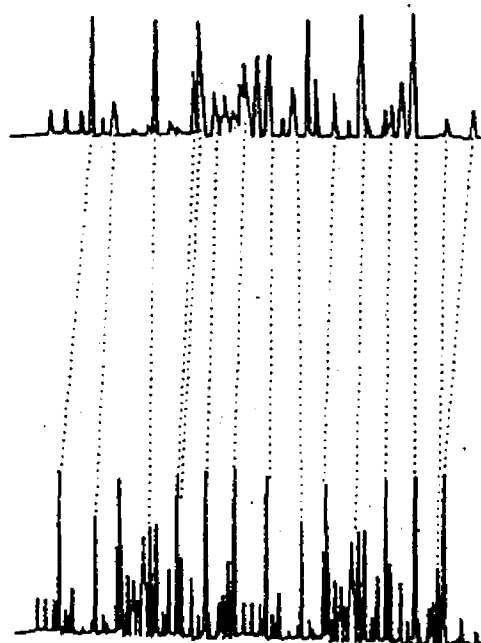
Comparison of gas chromatographic separation of perfume base using packed (top) and capillary columns (bottom). (Courtesy of Hewlett-Packard Co., Analytical Customer Training, Atlanta, GA.)

The solid support is most often diatomaceous earth (skeletons of algae) that has been purified, possibly chemically modified (e.g., silane treated), and then sieved to provide a definite mesh size (60/80, 80/100, or 100/120).

The liquid loading is usually applied to the solid support at 1–10% by weight of the solid support. While

the liquid coating can be any one of the approximately 200 available, the most common are silicone based phases (methyl, phenyl, or cyano substituted) and Carbowax (ester-based).

The liquid phase as well as the percent loading are determined by the analysis desired. The choice of liquid is typically such that it is of similar polarity as the analytes to be separated (see section 33.3.4.4 for more discussion). Loading influences time of analysis (retention time is proportional to loading), resolution (generally improved by increasing phase loading, within limits), and bleed. The liquid coatings are somewhat volatile and will be lost from the column at high temperatures (this is dependent upon the phase itself). This results in an increasing baseline (column bleeding) during temperature programming.

33.3.4.2 Capillary Columns

The capillary column is a hollow fused silica glass (<100 ppm impurities) tube ranging in length from 5 to 100 m. The walls are so thin, ca. 25 µm, that they are flexible. The column outer walls are coated with a polyamide material to enhance strength and reduce breakage. Column inner diameters are typically 0.1 mm (microbore), 0.2–0.32 mm (normal capillary), or 0.53 mm (megabore). Liquid coating is chemically bonded to the glass walls and internally crosslinked at phase thicknesses ranging from 0.1 to 5 µm.

33.3.4.3 Gas–Solid Chromatography

Gas–solid chromatography is a very specialized area of chromatography accomplished without using a liquid phase—the analyte interaction is with a synthetic polymer such as Poropak or Chromosorb (trade names of polymers based on vinyl benzene). This material has been applied both to packed and capillary columns. Separations usually involve water or other very volatile materials.

33.3.4.4 Stationary Phases

As many as 200 different liquid phases have been developed for GC. As GC has changed from packed to capillary columns, less stationary phases are now in use since column efficiency has substituted for phase selectivity (i.e., high efficiency has resulted in better separations even though the stationary phase is less suited for the separation). Now we find fewer than a dozen phases in common use (Table 33-3). The most durable and efficient phases are those based on poly-siloxane (—Si—O—Si—).

Stationary phase selection involves some intuition, knowledge of chemistry, and help from the column manufacturer and the literature. There are general

rules, such as choosing polar phases to separate polar compounds and the converse or phenyl-based column phase to separate aromatic compounds. However, the high efficiency of capillary columns often results in separation even though the phase is not optimal. For example, a 5% phenyl substituted methyl silicone phase applied to a capillary column will separate polar as well as nonpolar compounds and is a commonly used phase coating.

33.3.5 Detectors ·

There are numerous detectors available for GC, each offering certain advantages in either sensitivity (e.g., electron capture) or selectivity (e.g., atomic emission detector). The most common detectors are the flame ionization (FID), thermal conductivity (TCD), electron capture (ECD), flame photometric (FPD), and photoionzation (PID) detectors. The operating principles and food applications of these detectors are discussed below (more detail can be found in reference 23). The characteristics of these detectors are summarized in Table 33-4.

33.3.5.1 Thermal Conductivity Detector (TCD)

33.3.5.1.1 Operating Principles As the carrier gas passes over a hot filament (tungsten), it cools the filament at a certain rate depending on carrier gas velocity and composition. The temperature of the filament determines its resistance to electrical current. As a compound elutes with the carrier gas, the cooling effect on the filament is typically less, resulting in a temperature increase in the filament and an increase in resistance that is monitored by the GC electronics. Older style TCDs used two detectors and two matching columns; one system served as a reference and the other as the analytical system. Newer designs use only one detector (and column), which employs a carrier gas switching value to pass alternately carrier gas or column effluent though the detector (Fig. 33-5). The signal is then a change in cooling of the detector as a function of which gas is passing through the detector from the analytical column or carrier gas supply (reference gas flow).

The choice of carrier gas is important since differences between its thermal properties and the analytes determines response. While hydrogen is the best choice, helium is most commonly used since hydrogen is flammable.

33.3.5.1.2 Applications The most valuable properties of this detector are that it is universal in response and nondestructive to the sample. Thus, it is used in food applications where there is no other detector that will adequately respond to the analytes (e.g., water, permanent gases, CO, or CO,) or when the analyst wishes to

33-3
table Common Stationary Phases

Composition	Polarity	Applications[1]	Phases with Similar McReynolds Constants[2]	Temperature Limits[3]
100% dimethyl polysiloxane (gum)	Nonpolar	Phenols. hydrocarbons. amines. sulfur compounds. pesticides. PCBs	OV-1, SE-30	–60°C to 325°C
100% dimethyl polysiloxane (fluid)	Nonpolar	Amino acid derivatives. essential oils	OV-101, SP-2100	0°C to 280°C
5% phenyl, 95% dimethyl polysiloxane	Nonpolar	Fatty acids. methyl esters, alkaloids, drugs. halogenated compounds	SE-52, OV-23, SE-54	–60°C to 325°C
14% cyanopropylphenyl methyl polysiloxane	Intermediate	Drugs, steroids, pesticides	OV-1701	–200°C to 280°C
50% phenyl, 50% methyl methyl polysiloxane	Intermediate	Drugs, steroids, pesticides. glycols	OV-17	60°C to 240°C
50% cyanopropylmethyl, 50% phenylmethyl polysiloxane	Intermediate	Fatty acids, methyl esters, alditol acetates	OV-225	60°C to 240°C
50% trifluoropropyl polysiloxane	Intermediate	Halogenated compounds, + aromatics	OV-210	45°C to 240°C
Polyethylene glycol—TPA modified	Polar	Acids, alcohols, aldehydes, acrylates. nitrites. ketones	OV-351, SP-1000	60°C to 240°C
Polyethylene glycol	Polar	Free acids, alcohols. esters, essential oils. glycols, solvents	Carbowax 20M	60°C to 220°C

[1]Specific application notes from column suppliers provide information for choosing a specific column.
[2]McReynolds constants are used to group stationary phases together on the basis of separation properties.
[3]Stationary phases have both upper and lower temperature limits. Lower temperature limit is often due to a phase change (liquid to solid) and upper temperature limit to a volatilization of phase.

33-4
table Characteristics of Most Common Detectors for Gas Chromatography

Characteristic	Thermal Conductivity Detector	Flame Ionization Detector	Electron Capture Detector	Flame Photometric Detector	Photoionization Detector
Specificity	Very little; detects almost anything, including H₂O: called the "universal detector"	Most organics	Halogenated compounds and those with nitro or conjugated double bonds	Organic compounds with S or P (determined by which filter is used)	Depends on ionization energy of lamp relative to bond energy of solutes
Sensitivity limits	ca. 400 pg; relatively poor; varies with thermal properties of compound	10–100 pg for most organics; very good	0.05–1 pg; excellent	2 pg for S and 0.9 pg for P compounds; excellent	1–10 pg depending on compound and lamp energy; excellent
Linear range	10^4—poor; response easily becomes nonlinear	10^6–10^7; excellent	10^4—poor	10^4 for P; 10^3 for S	10^7—excellent

Vent

Vent

Switching   Column   Switching
Flow 1 (off)   Flow   Flow 2 (on)

Switching   Column   Switching
Flow 1 (on)   Flow   Flow 2 (off)
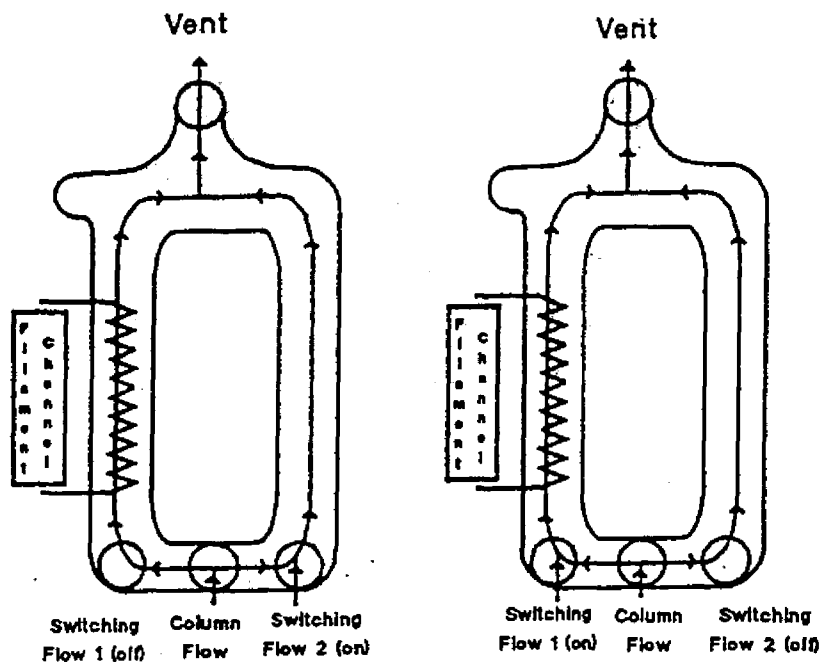
Pulse modulated single filament design

33-5 figure Schematic of the thermal conductivity detector. (Courtesy of Hewlett-Packard Co., Analytical Customer Training, Atlanta, GA.)

recover the separated compounds for further analysis (e.g., trap the column effluent for infrared, NMR, or sensory analysis). It does not find broad use because it is relatively insensitive, and often the analyst desires specificity in detector response to remove interfering compounds from the chromatogram.

for most food work. Thus, this detector is used for virtually all food analyses where a specific detector is not desired or sample destruction is acceptable (column eluant is burned in flame). This includes, for example, flavor studies, fatty acid analysis, carbohydrate analysis, sterols, contaminants in foods, and antioxidants.

33.3.5.2 Flame Ionization Detector (FID)

33.3.5.2.1 Operating Principles As compounds elute from the analytical column, they are burned in a hydrogen flame (Fig. 33-6). A potential (often 300 volts) is applied across the flame. The flame will carry a current across the potential which is proportional to the organic ions present in the flame from the burning of an organic compound. The current flowing across the flame is amplified and recorded. The FID responds to organics on a weight basis. It gives virtually no response to H_2O, NO_2, CO_2, H_2S and limited response to many other compounds. Response is best with compounds containing C—C or C—H bonds.

33.3.5.2.2 Applications The food analyst is most often working with organic compounds, which this detector responds well to. Its very good sensitivity, wide linear range in response (necessary in quantitation), and dependability make this detector the choice

33.3.5.3 Electron Capture Detector (ECD)

33.3.5.3.1 Operating Principles The electron capture detector contains a radioactive foil coating that emits electrons as it undergoes decay (Fig. 33-7). The electrons are collected on an anode, and the standing current is monitored by instrument electronics. As an analyte elutes from the GC column, it passes between the radioactive foil and the anode. Compounds that capture electrons reduce the standing current and thereby give a measurable response. Halogenated compounds or those with conjugated double bonds give the greatest detector response. Unfortunately this detector becomes saturated quite easily and thus has a very limited linear response range.

33.3.5.3.2 Applications In food applications, the ECD has found its greatest use in determining pesticide residues (see Chapter 20). The specificity and sensitivity of this detector make it ideal for this application.

Air
Inlet

Capillary Column
End-Position
(1-2 mm from Top of Jet)

Jet

H₂ Inlet
+
Make-Up
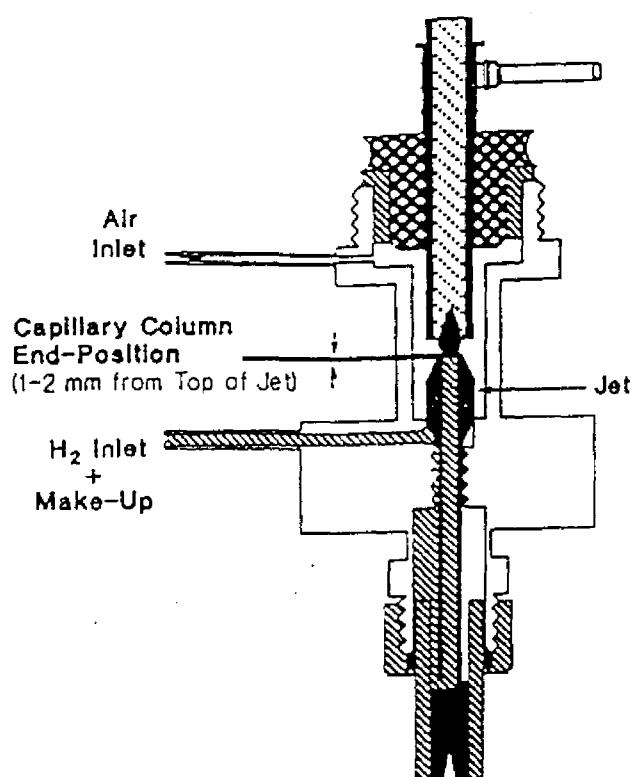
33-6
figure

Schematic of the flame ionization detector designed for use with capillary columns. (Courtesy of Hewlett-Packard Co., Analytical Customer Training, Atlanta, GA.)

Anode Purge

Vent

Anode

Plated Ni
Surface

Nickel Plating

Fused Silica
Liner

Makeup Gas
Adapter

Makeup Gas

Column
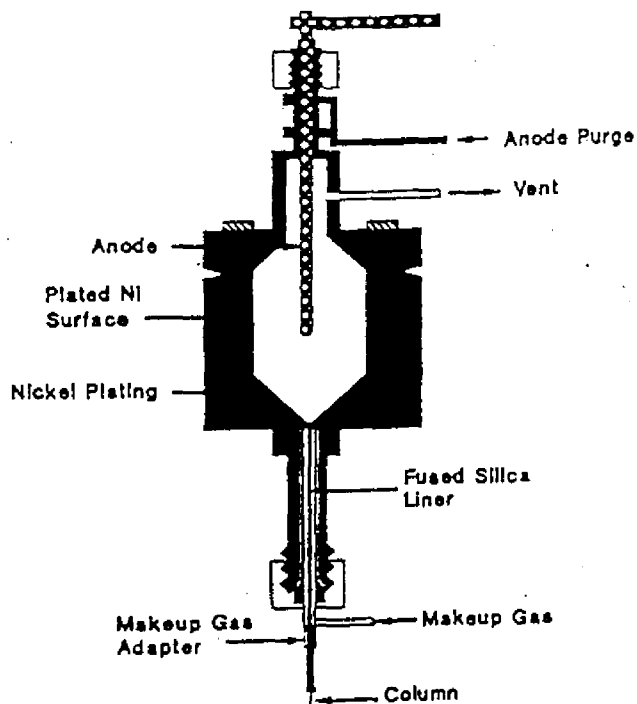
33-7
figure

Schematic of the electron capture detector. (Courtesy of Hewlett-Packard Co., Analytical Customer Training, Atlanta, GA.)

33.3.5.4 Flame Photometric Detector (FPD)

33.3.5.4.1 Operating Principles The FPD detector works by burning all analytes eluting from the analytical column and then measuring specific wavelengths of light that are emitted from the flame using a filter and photometer (Fig. 33-8). The wavelengths of light that are suitable in terms of intensity and uniqueness are characteristic of sulfur (S) and phosphorus (P). Thus this detector gives a greatly enhanced signal for these two elements (several thousandfold for S or P containing organic molecules versus non S or P containing organic molecules). Detector response to S containing molecules is nonlinear and thus quantification must be done with care.

33.3.5.4.2 Applications The FPD has found its major food applications in the determination of organophosphorus pesticides and volatile sulfur compounds in general. The determination of sulfur compounds has typically been in relation to flavor studies.

33.3.5.5 Photoionization Detector (PID)

33.3.5.5.1 Operating Principles The photoionization detector uses UV irradiation (usually 10.2 eV) to ionize analytes eluting from the analytical column (Fig. 33-9). The ions are accelerated by a polarizing electrode to a collecting electrode. The small current formed is magnified by the electrometer of the GC to provide a measurable signal.

This detector offers the advantages of being quite sensitive and nondestructive and may be operated in a selective response mode. The selectivity comes from being able to control the energy of ionization, which will determine the classes of compounds that are ionized and thus detected.

33.3.5.5.2 Applications The PID finds primary use in analyses for which excellent sensitivity is required from a nondestructive detector. This is most often a flavor application in which the analyst wishes to smell the GC effluent to determine the sensory character of the individual GC peaks. While this detector might find broader use, the widespread availability of the FID (which is suitable for most of the same applications) meets most of these needs.

33.3.5.6 Electrolytic Conductivity Detector (ELCD)

33.3.5.6.1 Operating Principles Compounds entering this detector are mixed with a reagent gas (oxidizing or reducing depending on the analysis) in a nickel reaction tube producing ionic species. These products are mixed with a deionized solvent, interfering ions are

Heat Filter

Photomultiplier Tube

Selective Filter

Thermocouple

Heater

Transfer Line

Capillary Column End-Position

Jet

Fused Silica Liner

Tube Housing

Detector Module
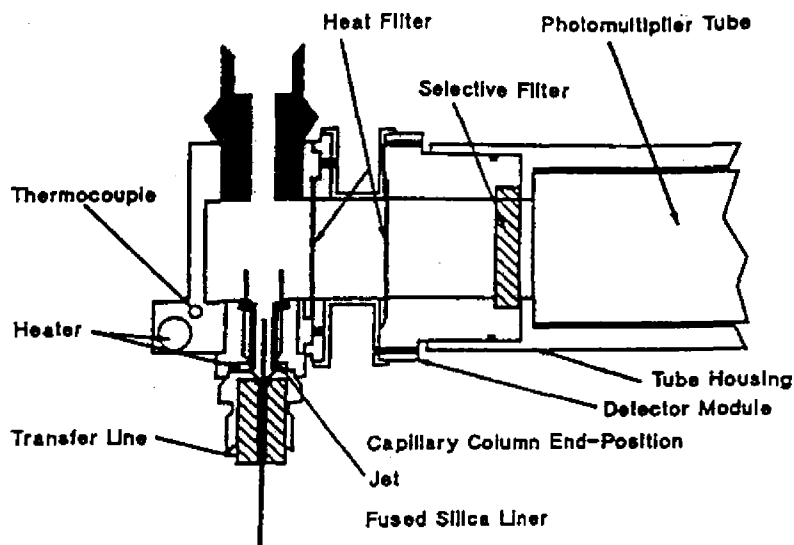
33-8 figure Schematic of the flame photometric detector. (Courtesy of Hewlett-Packard Co., Analytical Customer Training, Atlanta, GA.)

High-Energy UV Lamp

Exit

Polarizing Electrode

Collecting Electrode

Insulator

Reaction Cavity

To Amplifier (Electrometer)

Sample

$$\text{Compound} + \text{Photon} = \text{Compound}^{\oplus} + \text{Electron}^{\ominus}$$
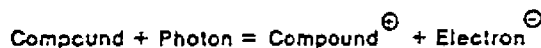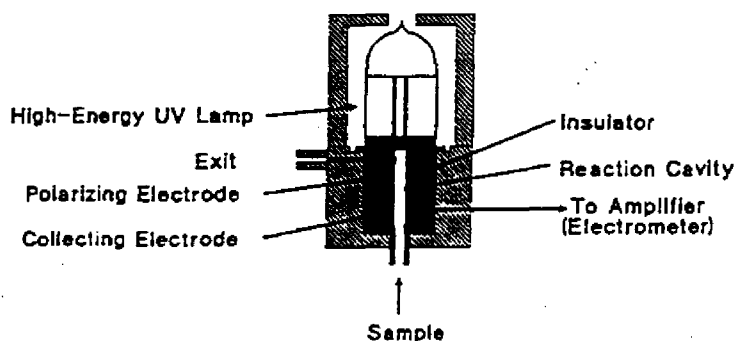
33-9 figure Schematic of the photoionization detector. (Courtesy of Hewlett-Packard Co., Analytical Customer Training, Atlanta, GA.)

scrubbed from the effluent, and the ionic analyte-transformation product is detected within the electrolyte conductivity cell. This detector can be used for the specific detection of sulfur-, nitrogen-, or halogen-containing molecules. For example, when operated in the nitrogen mode, analyte is mixed with H_2 gas and hydrogenated over a nickel catalyst at 850°C. Acidic hydrogenation products are removed from the effluent by passage through a $Sr(OH)_2$ trap and the NH_3 from the analyte passes to the conductivity cell where it is measured (23).

The ELCD is very selective and quite sensitive having detection limits of 0.1–1 pg of chlorinated compounds, 2 pg for sulfur, and 4 pg for nitrogen.

33.3.5.6.2 Applications This detector can be used in many applications where element specificity is desired. Examples would be pesticide, herbicide, nitrosamine, or flavor analysis.

33.3.5.7 Thermionic Detector (NPD)

33.3.5.7.1 Operating Principles The thermionic detector (also called the nitrogen phosphorus detector, NPD) is a modified flame ionization detector in which a nonvolatile ceramic bead is used to suppress the ionization of hydrocarbons as they pass through a low temperature fuel-poor hydrogen plasma. The ceramic bead is typically composed of rubidium which is

heated to 600–800°C. Most commonly this detector is used for the selective detection of nitrogen or phosphorus-containing compounds. It does not detect inorganic nitrogen or ammonia.

33.3.5.7.2 Applications This detector is primarily used for the measurement of specific classes of flavor compounds, nitrosamines, amines, and pesticides.

33.3.5.8 Hyphenated Gas Chromatographic Techniques

Hyphenated gas chromatographic techniques are those that combine GC with another major technique. Examples are GC–AED (atomic emission detector), GC–FTIR (Fourier transform infrared), and GC–MS (mass spectrometry). While all of the techniques are established methods of analysis in themselves, they become powerful tools when combined with a technique such as GC. GC provides the separation and the hyphenated technique the detector. GC-MS has long been known to be a most valuable tool for the identification of volatile compounds (see Chapter 29). The MS, however, may perform the task of serving as a specific detector for the GC by selectively focusing on ion fragments unique to the analytes of interest. The analyst can detect and quantify components without their gas chromatographic resolution in this manner. The same statements can be made about GC–FTIR (see Chapter 27). The FTIR can readily serve as a GC detector.

A relatively new combination is GC–AED. In this technique, the GC column effluent enters a microwave-generated helium plasma that excites the atoms present in the analytes. The atoms emit light at their characteristic wavelengths, and this emission is monitored using a diode ray detector similar to that used in HPLC. This results in a very sensitive and specific elemental detector.

33.4 CHROMATOGRAPHIC THEORY

33.4.1 Introduction

The principles of chromatographic separations and chromatographic theory are discussed in Chapter 31 (sections 31.3.3 and 31.3.4.3.3, respectively). GC may depend upon several types (or principles) of chromatography for separation. For example, size-exclusion chromatography is used in the separation of permanent gases such as N_2, O_2, and H_2. A variation of size exclusion is used to separate chiral compounds on cyclodextrin-based columns; one enantiomorphic form will fit better into the cavity of the cyclodextrin than will the other form, resulting in separation. Adsorption chromatography is used to separate very volatile polar

compounds (e.g., alcohols, water, and aldehydes) on porous polymer columns (e.g., Tenax® phase). Partition chromatography is the workhorse for gas chromatographic separations. There are over 200 different liquid phases that have been developed for gas chromatographic use over time. Fortunately, the vast majority of separations can be accomplished with only a few of these phases, and the other phases have fallen into disuse. GC depends not only upon adsorption, partition, and/or size exclusion for separation, but also upon solute boiling point for additional resolving powers. Thus, the separations accomplished are based on several properties of the solutes. This gives GC virtually unequaled resolution powers as compared to most other types of chromatography (e.g., HPLC, paper, or thin-layer chromatography).

A brief discussion of chromatographic theory will follow. The purpose of this additional discussion is to apply this theory to GC to optimize separation efficiency so that analyses can be done faster, less expensively, or with greater precision and accuracy. If one understands the factors influencing resolution in GC, one can optimize the process and gain in efficiency of operation.

33.4.2 Separation Efficiency

A good separation has narrow-based peaks and ideally, but not essential to quality of data, baseline separation of compounds. This is not always achieved. Peaks broaden as they pass through the column—the more they broaden, the poorer is the separation and efficiency. As discussed in Chapter 31 (section 31.3.4.3.3) a measure of this broadening is height equivalent to a theoretical plate (HETP). This term is derived from N, the number of plates in the column, and L, the length of the column. A good packed column might have N = 5000, while a good capillary column should have about 3000–4000 plates per meter for a total of 100,000–500,000 plates depending on column length. HETP will range from about 0.1 to 1 mm for good columns.

33.4.2.1 Carrier Gas Flow Rates and Column Parameters

Several factors influence column efficiency (peak broadening). These are related by the Van Deemter equation (1): (HETP values should be small.)

$$HETP = Au^{1\,3} + B/u + Cu \qquad [1]$$

where:

HETP = height equivalent to a theoretical plate
A = eddy diffusion
B = band broadening due to diffusion

u = velocity of the mobile phase

C = resistance to mass transfer

A is eddy diffusion; this is a spreading of the analytes in the column due to the carrier gas having various pathways or nonuniform flow (Fig. 33-10). In packed column chromatography, poor uniformity in solid support size or poor packing results in channeling and multiple pathways for carrier flow, which results in spreading of the analyte in the column. Thus, improved efficiency is obtained by using the high performance solid supports and commercially packed columns.

In capillary chromatography, the A term is relatively very small. However, as the diameter of the capillary column increases, the flow properties deteriorate, and band spreading occurs. The most efficient capillary columns have small diameters (0.1 mm), and efficiency decreases rapidly as one goes to megabore columns (Fig. 33-11). Megabore columns are only slightly more efficient than packed columns. While column efficiency increases as we go to smaller columns, column capacity decreases rapidly. Microbore columns are easily overloaded (capacity may be 1-5 ng per analyte), resulting again in poor chromatography. Thus,
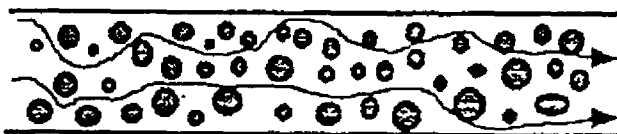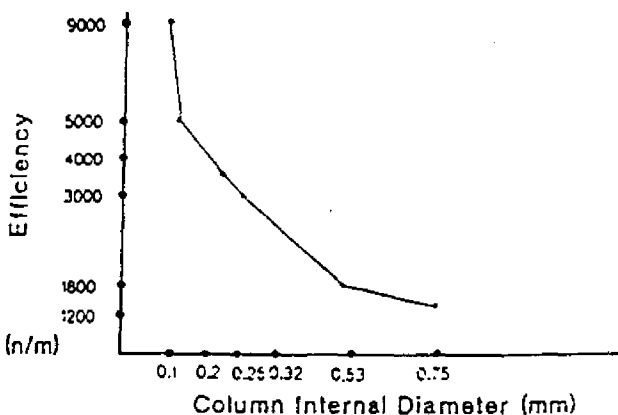
column diameter is generally chosen as 0.2-0.32 mm to compromise efficiency with capacity.

B is band broadening due to diffusion; solutes will go from a high to a low concentration (Fig. 33-12). The term u is velocity of the mobile phase. Thus, very slow flow rates result in large amounts of diffusion band broadening, and faster flow rates minimize this term.

C is resistance to mass transfer. If the flow (u) is too fast, the equilibrium between the phases is not established, and poor efficiency results. This can be visualized in the following way: If one molecule of solute is dissolved in the stationary phase and another is not, the undissolved molecule continues to move through the column while the other is retained. This results in band spreading within the column. Other factors that influence this term are thickness of the stationary phase and uniformity of coating on the phase support. Thick films give greater capacity (ability to handle larger amounts of a solute) but at a cost in terms of band spreading (efficiency of separation) since thick films provide more variation in diffusion properties in and out of the stationary phase (Fig. 33-13). Thus phase thickness is a compromise between maximizing separation efficiency and sample capacity (too much sample—overloading a column—destroys separation ability). Phase thicknesses of 0.25-1 µm are commonly used for most applications.

If the Van Deemter equation is plotted, the following figure results (Fig. 33-14). We see an optimum in flow rate due to the opposing effects of the B and the C terms. It should be noted that the GC may not be operated at a carrier flow velocity yielding maximum efficiency (lowest HETP). Analysis time is directly proportional to carrier gas flow velocity. If the analysis time can be significantly shortened by operating above the optimum flow velocity and adequate resolution is still

33-10
figure

Illustration of flow properties that lead to large eddy diffusion (term A).

33-11
figure

The influence of column diameter on column efficiency (plates/meter). (Courtesy of Hewlett-Packard. Analytical Customer Training, Atlanta, GA.)
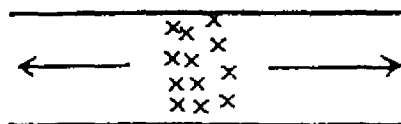
33-12
figure

Illustration of the diffusion term (B) influencing column efficiency.

is slower than
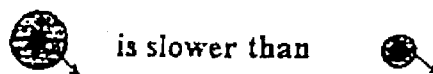
33-13
figure

Illustration of the mass transfer term (C) influencing column efficiency.

$$\text{HETP} = Au^{1/3} + \frac{B}{u} + Cu$$

HETP

MOLECULAR DIFFUSION B

C RESISTANCE TO MASS TRANSFER

A EDDY DIFFUSION

optimum u

u
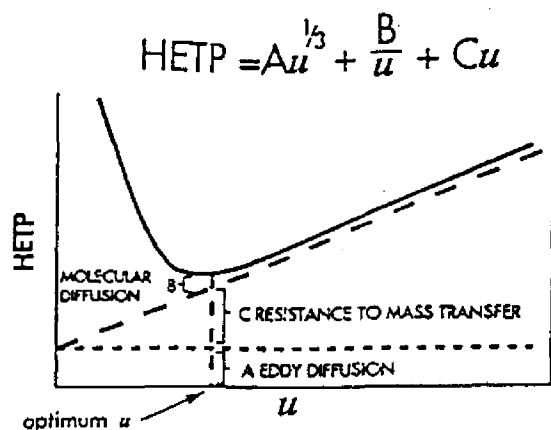
33-14 figure   The relationship between carrier gas flow rate (u) and column efficiency (HETP)-Van Deemter equation (optimum u is noted). (Courtesy of Hewlett-Packard Co., Analytical Customer Training, Atlanta, GA.)

HETP (mm)

C_i at 175°C
$K' = 4.96$
WCOT Column
OV-101 0.4 μm
25 m x 0.25 mm

N₂

He

H₂

10 20 30 40 50 60 70 80 90

Average Linear Velocity (cm/sec)

33-15 figure   Influence of carrier gas type and flow rate on column efficiency. (Courtesy of Hewlett-Packard Co., Analytical Customer Training, Atlanta, GA.)

obtained, velocities well in excess of optimum should be used.

33.4.2.2 Carrier Gas Type

The relationship between HETP and carrier gas flow velocity is strongly influenced by carrier gas choice (Fig. 33-15). Nitrogen is the most efficient but has an optimum at such a low flow velocity (long analysis time) that it is a poor choice since analysis times are much longer than required. Helium is the next best choice and is the most commonly used carrier. Hydrogen, however, is generally the best choice since it offers high efficiency but small dependency on flow velocity. One can operate the GC with very high flow velocities (short analysis time) and yet lose little in terms of separation efficiency. Hydrogen is not commonly used as a carrier gas because it is flammable.

33.4.2.3 Summary of Separation Efficiency

In summary, an important goal of analysis is to achieve the necessary separation in the minimum amount of time. The following factors should be considered:

1. In general, small diameter columns (packed or capillary) should be used since separation efficiency is strongly dependent on column diameter. While small diameter columns will limit column capacity, limited capacity can often be compensated for by increasing phase thickness. Increased phase thickness will also decrease column efficiency but to a lesser extent than increasing column diameter.

2. Lower column operating temperatures should

be used—if elevated column temperatures are required for the compounds of interest to elute, use a shorter column if resolution is adequate.

3. One should keep columns as short as possible (analysis time is directly proportional to column length—resolution is proportional to the square root of length).

4. Use hydrogen as the carrier gas if the detector permits. Some detectors have specific carrier gas requirements.

5. Operate the GC at the maximum carrier gas velocity that provides resolution.

The pyramid shown in Fig. 33-16 summarizes the compromises that must be made in choosing the analytical column and gas chromatographic operating conditions. One cannot optimize any given operating conditions and column choices to get one of these properties without compromising another property. For example, optimizing chromatographic resolution (small bore capillary diameter, thin phase coating, long column lengths, and slow or optimum carrier gas flow rate) will be at the cost of capacity (large bore columns and thick phase coating) and speed (thin film coating, high carrier gas flow velocities, and short columns). Capacity will be at a cost of resolution and speed etc. The choice of column and operating parameters must consider the needs of the analyst and the compromises involved in these choices.

33.5 APPLICATIONS OF GC

While some detail on the application of GC to food analyses has been presented in preceding chapters
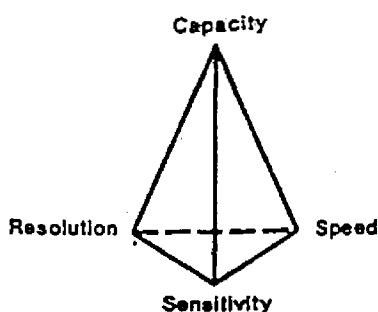
Relationships among column capacity, efficiency, resolution, and analysis speed. (Courtesy of Hewlett-Packard Co., Analytical Customer Training, Atlanta, GA.)

33-16 figure

33-17 figure Typical capillary gas chromatographic separation of residual volatiles in a food packaging film. [From (24), used with permission.]

(e.g., 11 and 20), a few additional examples will be presented below to illustrate separations and chromatographic conditions.

33.5.1 Residual Volatiles in Packaging Materials

Residual volatiles in packaging materials can be a problem both from health (if they are toxic) and quality standpoints (produce off flavors in the food). As the industry has turned from glass to polymeric materials, there have been more problems in this respect. GC is most commonly used to determine the residual volatiles in these materials.

The chromatograms presented in Fig. 33-17 were produced by steam distilling a food packaging film, extracting the volatiles from the distillate in an organic solvent, concentrating the solvent extract, and then chromatographing it on a capillary column (top chromatogram in Fig. 33-17) (24). The extreme complexity of the chromatogram required that the concentrate be further fractionated on silica gel and each fraction rechromatographed. The chromatograms labeled "cuts 1-5" are the chromatograms resulting from eluting the silica gel with: (1) hexane removing saturated hydrocarbons from the gel bed (cut 1); (2) 10% CH_2Cl_2/hexane removing the unsaturated and aromatic hydrocarbons (cut 2); (3) CH_2Cl_2 removing the ketones and aldehydes (cut 3); (4) methyl-t-butylether removing the acids, unsaturated ketones, and aldehydes (cut 4); and (5) alcohol removing the remaining polar volatiles (cut 5). One can see that the prefractionation of the extracted packaging volatiles greatly simplified the chromatography and permitted the researcher to focus on the volatiles responsible for the off odor in the packaging material.

33.5.2 Separation of Stereoisomers

GC has found extensive application in the separation of chiral volatile compounds in foods (e.g., d and l-car-

vone). Chiral separations are most commonly accomplished using cyclodextrin-based gas chromatographic columns. Cyclodextrins are molecules (6-, 7-, or 8-membered rings of glucose) that have an internal cavity of suitable dimensions to permit the inclusion of many small organic molecules. While optical isomers of molecules have virtually identical physical properties and thus they are difficult to separate by most chromatographic methods, they differ in spacial configuration. Stereoisomers of a given compound will be included in the cyclodextrin cavity of the gas chromatographic column to a lesser or greater extent as they flow through a cyclodextrin capillary column and become separated.
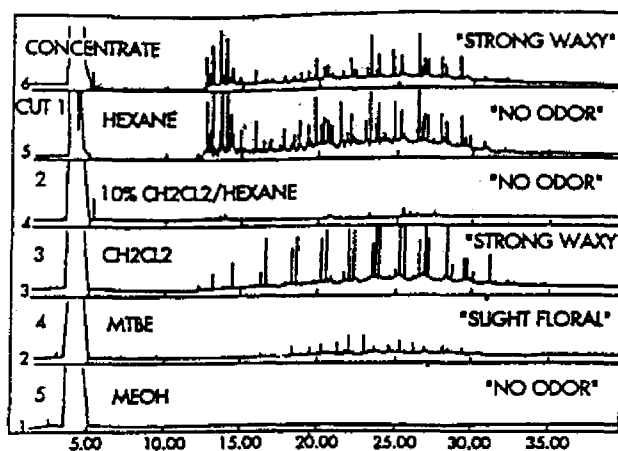
The chromatogram presented in Fig. 33-18 shows the separation of six stereoisomers of α and β irone (25). This separation was accomplished using an octakis (6-O-methyl-2,3-di-O-pentyl)-γ-cyclodextrin/ OV-17 capillary column.

33.5.3 Headspace Analysis of Ethylene Oxide in Spices

Ethylene oxide (ETO) is a highly volatile compound that has found use in the food industry as a fumigant for spices (26). It has been classified as a suspect human carcinogen and thus its residual concentration in spices is of concern. Because of its volatility, ETO is well suited to determination by GC.

Woodrow et al. (26) chose to use a headspace method for ETO determination. This is reasonable since ETO is very volatile, sensitivity is adequate, and headspace techniques are simple to perform. The method involved adding 1 g of ground spice to a 22-ml headspace vial (a vial that has a Teflon septum closure for sampling), adding internal standard (1-octanol), incubating the vial at 60°C for 20 min, and then removing and injecting ca. 1 ml of the headspace into the gas chro-

33.7 STUDY QUESTIONS

1. For each of the following methods to isolate solutes from food prior to GC analysis, describe the procedure, the applications, and the cautions in use of the method:
   a. headspace methods
   b. distillation methods
   c. solvent extraction
2. What is solid-phase extraction and why is it advantageous over traditional liquid-liquid extractions?
3. Why must sugars and fatty acids be derivatized before GC analysis, while pesticides and aroma compounds need not be derivatized?
4. Why is the injection port of a GC at a higher temperature than the oven temperature?
5. Differentiate packed columns from capillary columns (microbore and megabore) with regard to physical characteristics, column capacity, and column efficiency.
6. You are doing GC with a packed column and notice that the baseline rises from the beginning to the end of each run. Explain a likely cause for this increase.
7. The most common detectors for GC are TCD, FID, ECD,FPD, and PID. Differentiate each of these with regard to the operating principles. Also, indicate below which detector(s) fits the description given.
   a. least sensitive
   b. most sensitive
   c. least specific
   d. greatest linear range
   e. nondestructive to sample
   f. commonly used for pesticides
   g. commonly used for volatile sulfur compounds
8. What types of chromatography does GC rely upon for separation of compounds?
9. A fellow lab worker is familiar with HPLC for food analysis but not with GC. As you consider each component of a typical chromatographic system (and specifically the components and conditions for GC and HPLC systems), explain GC to the fellow worker by comparing and contrasting it to HPLC. Following that, state in general terms the differences among the types of samples appropriate for analysis by GC versus HPLC, and give several examples of food constituents appropriate for analysis by each. (See also Chapter 32.)

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Physical Properties of Foods

Rheological Principles for Food Analysis

Christopher R. Daubert and E. Allen Foegeding

34.1 INTRODUCTION

34.1.1 Rheological and Textural Properties

Food scientists often are confronted with the need to measure physical properties related to sensory texture and processing needs. These properties are determined by rheological methods. *Rheology is the study of the deformation and flow of matter.* This means that, for example, the flow of salad dressing from a bottle and the breaking of a candy bar are related to rheological properties of these materials. Rheological properties should be considered a component of the textural properties of foods, because the sensory detection of texture encompasses factors in addition to rheological properties. Rheological methods accurately measure "force," "deformation," and "flow," and it is up to the food scientist to determine how to apply this information. In this chapter, we (1) describe fundamental concepts pertinent to the understanding of the subject, (2) explain examples of rheological tests on common foods, and (3) provide an overview of more advanced rheological testing. A glossary has been included as section 34.6 to clarify and summarize rheological definitions throughout the chapter.

34.1.2 Fundamental and Empirical Methods

Rheological properties are determined by measuring force and deformation as a function of time. The difference between fundamental and empirical rheological methods is that, unlike the latter, the former accounts for the magnitude and direction of forces and deformations, placing restrictions on acceptable sample shapes and composition. Fundamental tests have the advantage in being based on known concepts and equations of physics. When sample composition or geometry is too complex to account for forces and deformations, empirical methods often are used. These methods follow a "cook and look" approach with the hopes of identifying a meaningful response. Empirical tests are of value if they correlate with a property of interest whereas fundamental tests determine true physical properties.

34.1.3 Basic Assumptions for Fundamental Rheological Methods

Two key assumptions are that the material is homogeneous and isotropic. Homogeneity implies a well mixed and compositionally similar material. This assumption is generally valid for fluid foods provided they are not a suspension of large particles such as vegetable soup. Foods such as milk, infant formula, and cream are considered homogeneous and isotropic. Homogeneity is more problematic in solid foods. For example, frankfurters without the skins can be considered homogeneous. When particle size is large enough to see, such as fat particles in some processed meats such as salami, one must determine if homogeneity is a valid assumption.

Isotropic means that the response to a deformation or force is the same no matter in which direction it is applied. In foods such as a steak, there are muscle fibers that make the material anisotropic (the response varies with the direction of the force or deformation).

34.2 FUNDAMENTALS OF RHEOLOGY

Rheology is concerned with how all materials respond to applied forces and deformations. Basic concepts of stress (force per area) and strain (deformation per length) are key to all rheological evaluations. Special constants of proportionality, called moduli, link stress with strain. With ideal solids, the material obeys Hooke's Law, which implies the stress is related directly to strain. With ideal fluids, the material follows Newtonian principles, and the proportionality constant is commonly referred to as viscosity, defined as an internal resistance to flow. These principles form the foundation for the entire chapter and are described in detail throughout this section.

34.2.1 Concepts of Stress

Stress (σ) is always a measurement of force. Defined as the force (F, Newtons) divided by an area (A, meters^2), stress is generally expressed with units of Pascals (Pa). To illustrate the notion of stress, conceptualize placing a water balloon on a table as opposed to placing it on the tip of a pair of scissors (Fig. 34-1). Obviously the scissors have a considerably smaller surface area, causing the stress or weight/unit area of contact to be larger compared with the stress of a balloon resting on a table
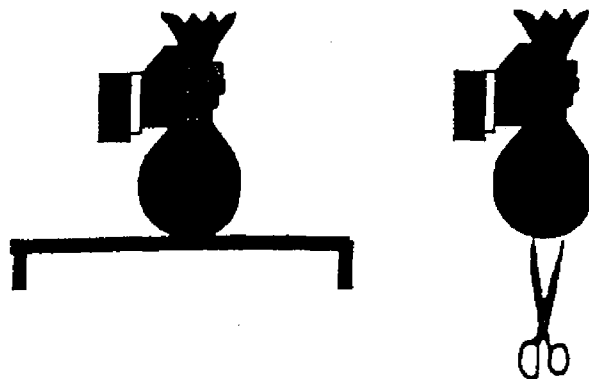
34-1 Concepts of stress.
figure
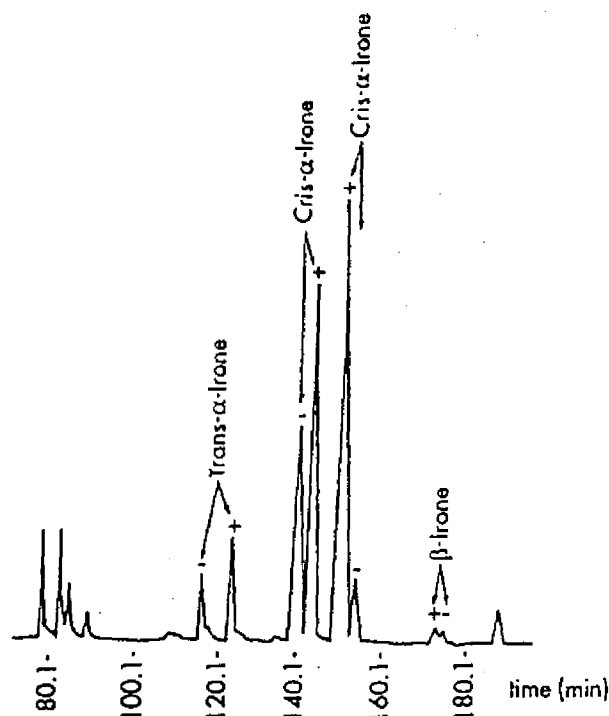
33-18 figure Enantiomeric analysis of irone sterioisomers in orris oil. [Reprinted from (25), p. 180, by courtesy of Marcel Dekker, Inc., New York.]

33-19 figure Headspace gas chromatographic analysis of ethylene oxide in spices. A is 3 μg pure ETO, B is 1 μg pure ETO, C is 3 μg ETO in spice, D is 1 μg-in spice, E is the pure spice. [From (26), used with permission.]

matograph. ETO was separated from other volatiles in the sample using a porous polymer capillary column (divinylbenzene homopolymer). Typical chromatograms of pure ETO, spice, and spice spiked with ETO are shown in Fig. 33-19.

33.6 SUMMARY

GC has found broad application in both the food industry and academia. It is exceptionally well suited to the analysis of volatile thermally stable compounds. This is due to the outstanding resolving properties of the method and the wide variety of detectors that can provide either sensitivity or selectivity in analysis.

Sample preparation generally involves the isolation of solutes from foods, which may be accomplished by headspace analysis, distillation, preparative chromatography (including solid-phase extraction), or extraction (liquid–liquid). Some solutes can then be directly analyzed, while others must be derivatized prior to analysis.

The gas chromatograph consists of a gas supply and regulators (pressure and flow control), injection port, column and column oven, detector, electronics, and a data recording and processing system. The analyst must be knowledgeable about each of these GC components: carrier and detector gases; injection port temperatures and operation in split, splitless, temperature programmed, or on-column modes; column choices and optimization (gas flows and temperature profile during separation); and detectors (TCD, FID, NPD, ECD, FPD, and PID). The characteristics of these GC components and an understanding of basic chromatographic theory are essential to balancing the properties of resolution, capacity, speed, and sensitivity.

Unlike most of the other chromatographic techniques, GC has reached the theoretical limits in terms of both resolution and sensitivity. Thus, this method will not change significantly in the future other than for minor innovations in hardware or associated computer software.

GC as a separation technique has been combined with AED, FTIR, and MS as detection techniques to make GC an even more powerful tool. Such hyphenated techniques are likely to continue to be developed and refined.

top. Although the force magnitude—the weight of the water balloon—is a constant value for each case, the final outcomes for this demonstration will be very different.

The direction of the force with respect to the surface area impacted determines the type of stress. For example, if the force is directly perpendicular to a surface, this is called normal stress and can be achieved in tension or compression. Should the force act in parallel to the sample surface, it is a shear stress. Examples of normal stresses include the everyday practice of chewing a piece of gum and the kneading of dough. During bread making, dough is continuously pressed and pulled until the proper consistency is achieved. Chewing a stick of gum involves the repetitive compression and extension of the material. Examples of shear forces are the spreading of butter over a slice of toast, the brushing of barbecue sauce on chicken, the stirring of a hot cup of cocoa, or the pumping of milk through a pasteurizer.

34.2.2 Concepts of Strain and Strain (Shear) Rate

When a stress is applied to a food, the food deforms or flows. Strain is a dimensionless quantity representing the relative deformation of a material. The direction of the applied stress with respect to the material surface will determine the type of strain. If the stress is (perpendicular) normal to a sample surface, the material will experience normal strain (ϵ). Foods show normal strains when they are compressed (compressive stress) or pulled apart (tensile stress).

Normal strain can be calculated at Cauchy (ϵ_c) or engineering strain. Considering a normal stress applied to a cylindrical specimen, the Cauchy strain is simply the length change (ΔL) divided by the initial lenght (L_i) (Fig. 34-2).

$$\epsilon_c = \frac{\Delta L}{L_i} \qquad [1]$$

Another normal strain calculation, known as the Hencky (ϵ_H) or true strain, is determined from an integration over the deformed length of the material.

$$\epsilon_H = \int_{L_i}^{L_i \cdot \Delta L} \frac{dL}{L} = \ln\left(1 + \frac{\Delta L}{L_i}\right) \qquad [2]$$

According to Steffe (1), true strain is more applicable to larger deformations such as may occur in texture testing. Strain calculations result in negative valves for compression and positive values for extension (tensile). Rather than expressing a negative strain, it is typical to record the absolute value of the strain and denote the compression test mode.

$$\epsilon_c = -0.05 \equiv 0.05_{compression} \qquad [3]$$
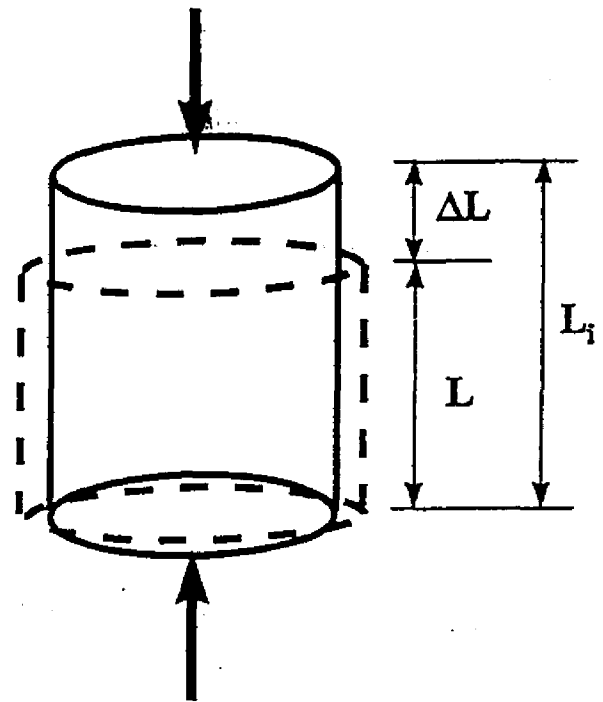
34-2 figure   Normal strain in a cylinder.
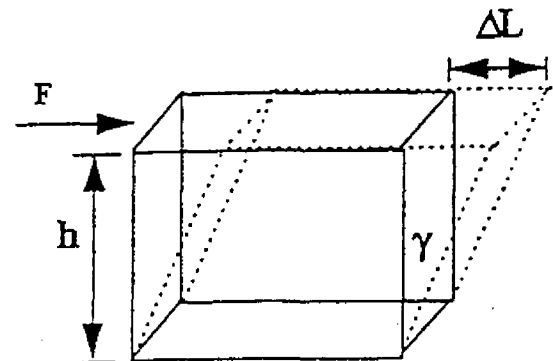
34-3 figure   Shear strain in a cube.

On the other hand, when a sample encounters a shear stress—such as the pumping of tomato paste—a shear strain (γ) is observed. Figure 34-3 shows how a sample deforms when a shear stress is applied. Shear strain is determined from applications of geometry as

$$\tan(\gamma) = \frac{\Delta L}{h} \qquad [4]$$

or

$$\gamma = \tan^{-1}\left(\frac{\Delta L}{h}\right) \qquad [5]$$

where h is the specimen height. For simplification, dur-

ing exposure to small strains, the angle of shear may be considered equal to the shear strain.

$$\tan(\gamma) = \gamma \qquad [6]$$

When the material is a liquid, this approach for strain quantification is difficult. As coffee is stirred, water is pumped, or milk is homogenized, these fluids all are exposed to shear and display irrecoverable deformation. Due to permanent deformation of sheared fluids, it is complicated to characterize the degree of shear strain. Therefore, a shear (strain) rate $(\dot{\gamma})$ is typically used to quantify strain during fluid flow. Shear rate is the degree of deformation with respect to time, with common units of sec^{-1}.

To further the explanation of shear rate, consider a fluid filling the gap between two moveable, parallel plates separated by a known distance, h, as illustrated in Fig. 34-4. Now, set one plate in motion with respect to the other at a constant horizontal velocity, U.

$$\gamma = \frac{\Delta L}{h} \qquad [7]$$

$$\frac{d(\gamma)}{dt} = \frac{d\left(\frac{\Delta L}{h}\right)}{dt} = \dot{\gamma} \qquad [8]$$

$$\dot{\gamma} = \frac{1}{h}\frac{d}{dt}(\Delta L) \qquad [9]$$

$$\dot{\gamma} = \frac{U}{h} \qquad [10]$$

The shear strain rate for this system can be approximated as the quotient of the plate velocity divided by the fluid gap height producing units of sec^{-1}. This shear strain rate may be more easily understood through a deck of cards analogy. Imagine a stack of playing cards, with each card representing an infinitely thin layer of fluid. When the top card is stroked with some force, the entire deck deforms to some degree proportional with the magnitude of the force. This type of straining is commonly called simple shear and may be defined as
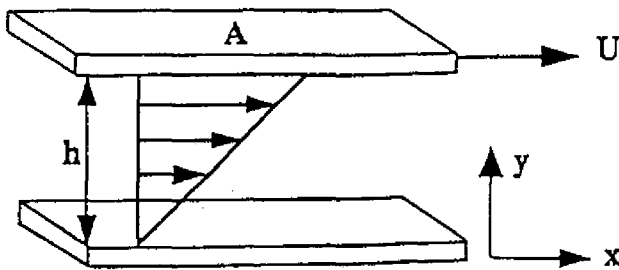
figure
Shear flow between parallel plates.

laminar deformation in a plane parallel with an applied force.

34.2.3 Solids—Elastic and Shear Moduli

Hooke's Law states that when a solid material is exposed to a stress, it experiences an amount of deformation or strain proportional to the magnitude of the stress. The constants of proportionality, used to equate stress with strain, are called moduli.

$$\text{Stress } (\sigma) \propto \text{Strain } (\varepsilon \text{ or } \gamma) \qquad [11]$$

$$\text{Stress} = \text{Modulus} \cdot \text{Strain} \qquad [12]$$

If a normal stress is applied to a sample, the proportionality constant is known as elastic modulus (E), sometimes called Young's modulus.

$$\sigma = \frac{F}{A} = E\varepsilon \qquad [13]$$

Likewise, if the stress is shear, the constant is the shear modulus (G).

$$\sigma = G\gamma \qquad [14]$$

The moduli are inherent properties of the material and may be used as indicators of quality. Moduli of select foods and materials are seen in Table 34-1.

34.2.4 Fluid Viscosity

In the case of the simplest kind of fluid, the viscosity is constant for all shear rates and Newton's postulate is obeyed. This postulate reasons that if the shear stress is doubled, the velocity gradient (shear strain rate) within the fluid is doubled. For fluids, strain is measured in terms of shear rate, and the shear stress may be expressed as some function of shear rate and viscosity. Viscosity is an indication of the internal resistance of a fluid to flow. For Newtonian fluids, the viscosity function is constant and called the coefficient of viscosity or Newtonian viscosity (μ).

34-1 table Elastic and Shear Moduli for Common Materials

Material	E, Elastic Moduli (Pa)	G, Shear Moduli (Pa)
Apple	1.0×10^{7}	0.38×10^{7}
Potato	1.0×10^{7}	0.33×10^{7}
Spaghetti, dry	0.27×10^{10}	0.11×10^{10}
Glass	7.0×10^{10}	2.0×10^{10}
Steel	25.0×10^{10}	8.0×10^{10}

$$\sigma = \mu \dot{\gamma} \qquad [15]$$

However, for most liquids the viscosity is not constant, but rather a function of the shear rate, and the material is non-Newtonian. A function called the apparent viscosity (η) is defined as the shear-dependent viscosity. Mathematically, the apparent viscosity function is the result of the shear stress divided by the shear rate.

$$\eta = f(\dot{\gamma}) = \frac{\sigma}{\dot{\gamma}} \qquad [16]$$

Table 34-2 displays Newtonian viscosities for some common items at 20°C (1, 2). These viscosities are for a specific temperature. Typically viscosity decreases as temperature increases.

34.2.5 Fluid Rheograms

The overall flow behavior of a food is a physical property that can be determined by rheological methods. A rheogram is a graphical representation of the flow behavior, showing the relationship between stress and strain or shear rate. Much can be learned from inspection of rheograms. For example, if a plot of shear stress versus shear rate results in a straight line passing through the origin, the material is a Newtonian fluid, with the slope of the line equaling the Newtonian viscosity (μ), shown in Fig. 34-5. Many common foods exhibit this ideal response, including water, milk, vegetable oils, and honey.

Many fluid foods do not show Newtonian flow behavior. The flow changes with shear rate (i.e., mixing speed) or with time at a constant shear rate. Time-independent deviation from ideal Newtonian behavior will cause the relationship between shear stress and shear rate to be nonlinear. Should the viscosity diminish as shear rate increases, the material is referred to as shear thinning or pseudoplastic. Examples of pseudoplastic food items are applesauce and many pie fillings.

On the other hand, if the material responds in a thickening fashion, i.e., the viscosity increases with

$$\mu_1 < \mu_2 < \mu_3$$
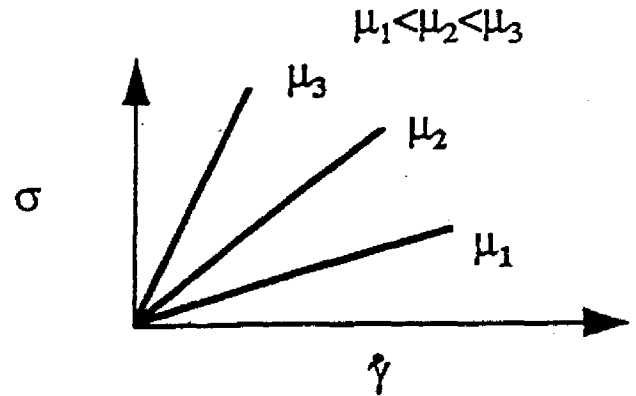
34-5 Rheogram for three Newtonian fluids.
figure

shear rate, the sample is called shear thickening or dilatent. Corn starch slurries are well known for dilatent behavior.

Pseudoplasticity and dilatency are time-independent properties. Materials that thin and thicken with time are known as thixotropic and rheopectic liquids, respectively. These fluids are easily detected by monitoring the viscosity at a constant shear rate with respect to time. If pumpkin pie filling is mixed at a constant speed, the material thins (thixotropy) with time due to the destruction of weak bonds linking the molecules. Table 34-3 summarizes the terminology for time dependent and independent responses.

Many fluids do not flow at low magnitudes of stresses. In fact, a certain catsup once staked numerous marketing claims on the "anticipation" of flow from the bottle. Often, additional force was applied to the catsup container to expedite the pouring. The minimum force, or stress, required to initiate flow is known as a yield stress (σ_0). Because Newtonian fluids require the stress–shear rate relationship to be a continuous straight line passing through the origin, any material with a yield stress is non-Newtonian. A few foods possessing yield stresses are catsup, mayonnaise, and salad dressing.

Many foods are designed to include a certain yield stress. For example, if melted cheese did not have a yield stress, the cheese would flow off a cheeseburger or pizza. If salad dressings flowed at the lowest of ap-

34-2 Newtonian Viscosities for Common
table Materials at 20°C

Material	Viscosity, μ (Pa sec)
Honey	11.0
Rapeseed oil	0.163
Olive oil	0.084
Cottonseed oil	0.070
Raw milk	0.002
Water	0.001
Air	0.0000181

Adapted from (1, 2).

34-3 Summary of
table Shear-Dependent Terminology

	Time Independent	Time Dependent
Thinning	Pseudoplastic	Thixotropic
Thickening	Dilatent	Rheopectic

plied stresses, the force of gravity would cause the dressing to run off the salad leaves. There are many fascinating rheological features of foods that many consumers may never consider!

34.2.6 Viscoelasticity

Numerous materials simultaneously display solidlike (elastic) and fluidlike (viscous) behavior. This phenomenon is called viscoelasticity. There are viscoelastic fluids and viscoelastic solids. In fact, most foods are viscoelastic. Viscoelasticity complicates the issue of characterizing fluid viscosity and solid moduli. To identify viscoelastic properties, one must consider the relative time frame required to record a measurement.

It must be remembered that one must deform or flow a sample to determine rheological properties. This requires some amount of time, and during that time the material will be adjusting to the deformation, meaning that what we measure depends on how rapidly we measure. Take for example moving your hand through water. The faster (shear rate) you move your hand, the more resistance you feel. The faster you move your hand the more water feels like a solid than a fluid. Note that the water and surface area (i.e., your hand) have not changed but the shear rate has increased. This concept is very important to viscoelasticity.

The Deborah number (De) is a way to relate the time it takes for a material to flow and the measurement time (i.e., the time it takes the instrument to make a reading). The term comes from the Old Testament of the Bible where Deborah said "The mountains flowed before the Lord." The concept being that everything will flow—you just have to watch long enough to see it! The Deborah number relates the characteristic response time of the material (t) to the characteristic time of the deformation process (T).

$$De = t/T \qquad [17]$$

Small Deborah numbers (<<1) represent fluidlike behavior while large numbers (>>1) represent solidlike behavior. What this means to rheological measurement of viscoelastic foods is that the relative amount of viscous and elastic behavior is determined by the rate of the deformation process.

34.3 RHEOLOGICAL FLUID MODELS

Once you have the data for shear stress and shear rate, you can use rheological models to gain a greater understanding of the flow response. Rheological models are mathematical expressions relating shear stress to shear strain rate providing a "flow fingerprint" for

fluid foods. In addition, the models permit prediction of rheological behavior over a wide range of operating conditions.

34.3.1 Herschel–Bulkley Model

For most practical purposes, the Herschel–Bulkley model can account for the steady-state rheological performance of many fluid foods.

$$\sigma = \sigma_0 + K\dot{\gamma}^n \qquad [18]$$

K and n represent material constants called the consistency coefficient and flow behavior index, respectively. The flow behavior index provides an indication of Newtonian or non-Newtonian flow, providing the material has no yield stress. Table 34-4 illustrates how the Herschel–Bulkley model is used to identify specific flow characteristics.

The following models are considered as simple modifications to the Herschel–Bulkley model.

34.3.2 Newtonian Model [$n = 1$; $K = \mu$; $\sigma_0 = 0$]

For Newtonian fluids, Equation [18] is manipulated with the flow behavior index (n) equaling 1.0 and the consistency coefficient (K) equaling the Newtonian viscosity (μ).

$$\sigma = 0 + \mu\dot{\gamma}^1 \qquad [19]$$

or

$$\sigma = \mu\dot{\gamma} \qquad [20]$$

34.3.3 Power Law Model [$\sigma_0 = 0$]

Power law fluids show no yield stress (σ_0) and a nonlinear relationship between shear stress and shear rate. Pseudoplastic and dilatent fluids are considered power

34-4 table Manipulation of Herschel–Bulkley Model to Describe Flow Behavior

Fluid Type	σ_0	n
Newtonian	0	1.0
Non-Newtonian		
Pseudoplastic	0	<1.0
Dilatent	0	>1.0
Yield Stress	>0	any

σ_0 = Yield stress; n = flow behavior index.

law fluids, each with different ranges for flow behavior
index values, refer to Table 34-1.

$$\sigma = 0 + K\dot{\gamma}^n \qquad [21]$$

or

$$\sigma = K\dot{\gamma}^n \qquad [22]$$

34.3.4 Bingham Plastic Model [n = 1; $K = \mu_{pl}$]

Bingham plastic materials have a distinguishing fea-
ture—they have a yield stress. Once flow is established,
the relationship between shear stress and shear rate is
linear—explaining why n = 1.0 and K is constant value
known as the plastic viscosity, μ_{pl}. Caution: The plastic
value is *not* the same as the apparent viscosity (η) or
the Newtonian viscosity (μ)!

$$\sigma = \sigma_0 + \mu_{pl}\dot{\gamma}^1 \qquad [23]$$

or

$$\sigma = \sigma_0 + \mu_{pl}\dot{\gamma} \qquad [24]$$

34.4 RHEOMETRY

Rheometers are devices used to determine rheological
properties of materials. Relationships between shear
stress and shear rate are derived from physical values
of system configurations, pressures, flow rates, and
other applied forces.

34.4.1 Tube Viscometry

Tube viscometers consider pressure-driven flow
through a pipe or capillary. Rheological properties may
be derived from a pressure drop across a specified tube
length of known cross-sectional area and the volumet-
ric flow rate through the tube. Steffe (1) provides addi-
tional information regarding tube viscometry.

U-tube viscometers are gravity-driven instru-
ments constructed from glass. Common types of U-
tubes include Ostwald and Cannon–Fenske Viscome-
ters (Fig. 34-6). These viscometers each contain a
narrow capillary section of tube, serving as the limiting
factor for flow. The fluid is timed as it passes through
specific points etched in the glass. Depending upon the
capillary diameter, a series of factors are provided to
relate the resulting time to a kinematic viscosity. Kine-
matic viscosity is the Newtonian viscosity divided by
the material density. A limiting factor for these vis-
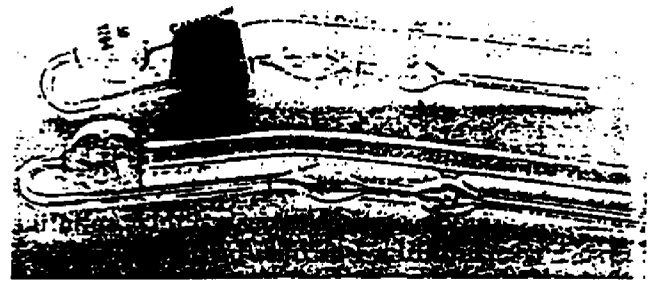
34-6 Photo of a U-tube viscometer.
figure

cometers is that they are ineffective for non-Newtonian
fluids. However, these viscometers are commonly used
at very low concentrations of food hydrocolloids for
molecular weight prediction.

As the name indicates, high pressure capillary
viscometers require a driving force beyond gravity.
A pump or cross head may supply an elevated pres-
sure to force material through a capillary. Rheological
parameters may be derived from a pressure drop
across the capillary section and the volumetric flow
rate through the system. This system is used typically
for very viscous materials or to analyze rheological
properties at high shear rates.

34.4.2 Rotational Viscometry

For fluids, the primary mode for rheological measure-
ment in the food industry is rotational viscometry, pro-
viding rapid and fundamental information. Rotational
viscometry involves a known test fixture (geometrical
shape) in contact with a sample, and through some
mechanical, rotational means, the fluid is sheared by
the fixture. Primary assumptions are made for the
development of constitutive equations (relationships
between shear stress and rate), and a few include:

- *Laminar Flow.* Laminar flow is synonymous
  with streamline flow. In other words, if we were
  to track velocity and position of a fluid molecule
  through a horizontal pipe, the molecule path
  line would be only in the horizontal direction.
- *Steady State.* Steady state means time-indepen-
  dent effects.
- *No-Slip Boundary Condition.* When the test fix-
  ture is immersed in the fluid sample, the wall of
  the fixture and the sample container serve as
  boundaries for the fluid. This condition assumes
  at whatever speed either boundary is moving,
  an infinitely thin layer of fluid immediately

adjacent to the boundary is moving at the exact same velocity.

Rotational rheometers may operate in two modes: steady shear or oscillatory. At this point we will consider steady shear rotational viscometry, and oscillatory viscometry will be described in a later section. Steady shear is a condition in which the sheared fluid velocity, contained between the boundaries, remains constant at any single position. Two test fixtures most often used in steady shear rotational viscometry are concentric cylinder and the cone and plate (Fig. 34-7 and Fig. 34-11).

34.4.2.1 Concentric Cylinders

This apparatus consists of a cylindrical fixture shape, commonly called a bob with radius R_b, suspended from a torque (M) measuring device and immersed in a sample fluid contained in a slightly larger cylinder, referred to as the cup with radius R_c (see Fig. 34-8). Torque is an action that generates rotation about an axis and is the product of a force (F) and the perpendicular distance (r), called the moment arm, to the axis of rotation. The principles involved can be described relative to changing tires on a car. To loosen the lugnuts, a larger tire iron is often required. Essentially, this longer tool increases the moment arm, resulting in a greater torque about the lugnut. Even though you are still applying the same force on the iron, the longer device provides greater torque!

To derive rheological data from experiments, expressions for shear stress and shear rate must be determined. Shear stress at the surface of the bob (σ_b) may be calculated from a force balance as

$$\sigma_b = \frac{M}{2\pi h R_b^2} \qquad [25]$$

Therefore, to determine shear stress, all we need to know is the bob geometry (h and R_b) and torque response (M) of the fluid on the measuring sensor.

A simple shear approximation is used often to predict a shear rate at the bob surface and assumes a
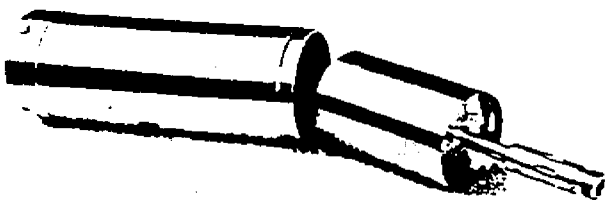
34-7 Photo of cup and bob test fixtures for rheological measurements.

SIDE VIEW   **TOP VIEW**
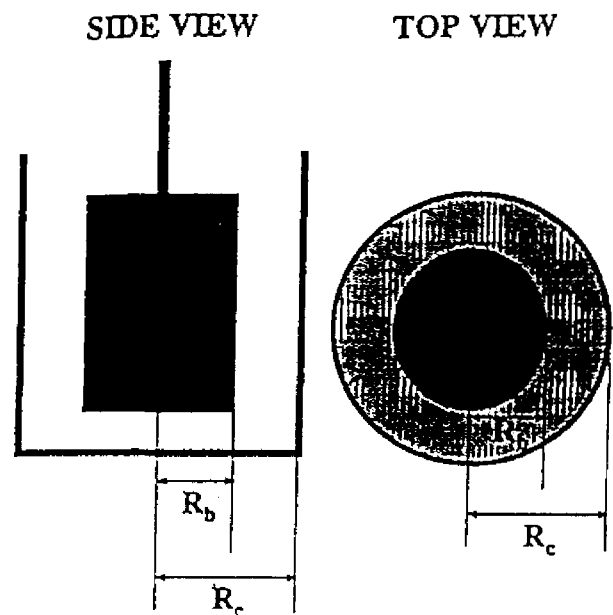
34-8 Concentric cylinder geometry.

constant shear rate across the fluid gap. This approximation is valid for small gap widths where $R_c/R_b \leq 1.1$.

$$\dot{\gamma}_b = \frac{\Omega R_b}{R_c - R_b} \qquad [26]$$

Ω is the angular velocity typically expressed in radians per second. Converting units of revolutions per minute (rpm) to radians per second is simply achieved by multiplying by $2\pi/60$. The following example converts from rpm to radians/second.

$$\frac{10 \text{ revolutions}}{1 \text{ min}} \times \frac{2\pi \text{ radians}}{1 \text{ revolution}}$$

$$\times \frac{1 \text{ min}}{60 \text{ sec}} = 1.047 \text{ radians/sec} \qquad [27]$$

One of the most common rheological devices found in the food industry is the Brookfield viscometer (Fig. 34-9). This simple apparatus uses a spring as a torque sensor. The operator selects a rotational speed (rpm) of the bob, attached to the spring. Once the rotational speed is converted to an angular velocity, the simple shear approximation may be used to calculate a shear rate. As the bob moves through the sample fluid, the viscosity impedes free rotation, causing the spring to wind. The degree of spring windup is a direct reflection of the torque magnitude (M), used to determine a shear stress at the bob surface.

From these data, a rheogram can be created showing shear stress (σ) versus shear rate ($\dot{\gamma}$). The importance of rheograms has been discussed, with the primary significance being apparent viscosity determination

34-5 **table** Rheological Data of Tomato Ketchup Collected Using a Concentric Cylinder Test Fixture

rpm	Torque (N m)	Shear Rate (sec⁻¹)	Shear Stress (Pa)	Apparent Viscosity (Pa sec)
1.0	0.00346	2.09	22.94	10.98
2.0	0.00398	4.19	26.39	6.30
4.0	0.00484	8.38	32.10	3.83
8.0	0.00606	16.76	40.18	2.40
16.0	0.00709	33.51	47.02	1.40
32.0	0.00848	67.02	56.23	0.84
64.0	0.01060	134.04	70.29	0.52
128.0	0.01460	268.08	96.82	0.36
256.0	0.01970	536.16	130.63	0.24
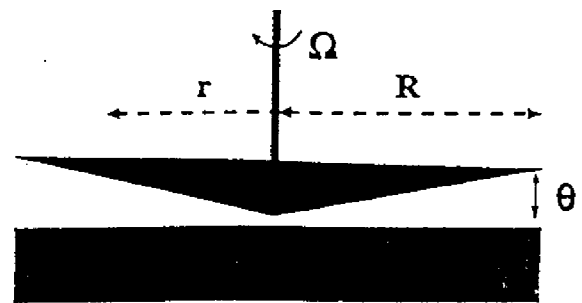
h: gap height at any radial location (r) of the cone.

34-10 **figure** Cone and plate configuration.

34-9 **figure** Photo of Brookfield viscometer.

(Equation [16]). The following tomato ketchup data in Table 34-5 were collected with a standard cup and bob system ($R_c = 21$ mm, $R_b = 20$ mm, and $h = 60$ mm). Using Equations [16], [25], and [26] you should verify the results.

34.4.2.2 Cone and Plate

Another popular system for rotational measurement is the cone and plate configuration (Figs. 34-10 and 34-11). Its special design permits the shear stress and shear rate to remain constant at any radial location of the gap. Quality of test results is best when the cone angle is small ($\theta \approx 1°$). Large errors are encountered when the gap is improperly set or not well maintained.

The shear stress may be determined for a cone and plate configuration as:

$$\sigma = \frac{3M}{2\pi R^3} \qquad [28]$$

34-11 **figure** Photo of cone and plate test fixtures for rheological measurements.

The shear rate is calculated as

$$\dot{\gamma} = \frac{r\Omega}{r \tan\theta} = \frac{\Omega}{\tan\theta} \qquad [29]$$

Like shear stress, the shear rate is constant throughout the gap—one of the primary advantages of this test fixture.

34.4.2.3 Experimental Procedure for Steady Shear Rotational Viscometry

34.4.2.3.1 Test Fixture Selection Many considerations go into the decision of selecting a fixture for a rheological test. To simplify the process, the information in Table 34-6 should be considered (3).

34.4.2.3.2 Speed (Shear Rate) Selection When performing a rheological test, it is crucial to have an understanding of the process for which the measurement is being performed. From the earlier example of tomato ketchup, the apparent viscosity continuously decreases, exhibiting shear thinning behavior, as the shear rate is increased. How would one report a viscosity? To answer that question, the process must be addressed. For example, if a viscosity for molten milk chocolate is required for pipeline design and pump specification calculations, a shear rate for this process should be known. All fluid processes administer a certain degree of shear on the fluid, and a good food scientist will consider the processing shear rate for proper rheological property determination. Barnes et al. (4) have prepared a list of common shear rates for typical processes, many of which are shown in Table 34-7.

34.4.2.3.3 Data Collection Once the test fixture and shear rate ranges have been selected, the experiment can begin. Record values of torque for each viscometer speed.

34.4.2.3.4 Shear Calculations Values for shear stress and shear rate are calculated based on test fixture, fixture geometry, and angular velocity.

34.4.2.3.5 Model Parameter Determination Shear stress and shear rate can now be inserted into various rheological models previously described in section 34.3. Rheological model parameters such as viscosity (μ, η, μ_{pl}), yield stress (σ_0), consistency coefficient (K),

34-7 table	Predicted Shear Rates for Typical Food Processes

Process	Shear Rate (sec^{-1})
Sedimentation of powders in a liquid	$10^{-6}-10^{-4}$
Draining under gravity	$10^{-1}-10^{1}$
Extruding	$10^{0}-10^{2}$
Chewing and swallowing	$10^{1}-10^{2}$
Coating	$10^{1}-10^{2}$
Mixing	$10^{1}-10^{3}$
Pipe flow	$10^{0}-10^{3}$
Spraying and brushing	$10^{3}-10^{4}$

Reprinted from Barnes, H.A., Hutton, J.F., and Walters, K. *An Introduction to Rheology*, Copyright 1989, with kind permission from Elsevier Science-NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, the Netherlands.

and the flow behavior index (n) now may be analyzed for an even greater understanding of the flow behavior of the material. For example, one may want to know: Does the material have a yield stress? Is the material shear thinning or shear thickening? Answering these and similar questions gives the food scientist a greater command of the behavior of the material for process design or quality determination.

34.4.3 Solids—Compression, Extension, and Torsion (Shear) Analysis

The rheological properties of solid foods are measured by compressing, extending, or twisting the material. This can be accomplished by two general approaches called small strain or large strain testing. In small strain tests the goal is to apply the minimal amount of strain or stress required to measure the rheological behavior while at the same time preventing (or at least minimizing) damage to the sample. The goal in large strain or fracture tests is the opposite. Samples are

34-6 table	Advantages and Disadvantages of Rotational Viscometry Attachments

Rotational Geometry	Advantages	Disadvantages
Concentric cylinder	• Good for low viscosity fluids • Good for suspensions • Large surface area increases sensitivity at low shear rates	• Potential end effects • Large sample required
Cone and plate	• Constant shear stress and shear rate in gap • Good for high shear rates • Good for medium and high viscosity samples • Small sample required • Quick-and-easy cleanup	• Large particles interfere with sensitivity • Potential edge effects • Must maintain constant gap height

deformed to an extent at which the food matrix is damaged or fractured. Small strain tests are used when one wants to understand properties of a food matrix, whereas large strain tests give an indication of sensory texture or product durability.

34.4.3.1 Large Strain Testing

Compression and tension (i.e., extension) tests can be used to determine large strain and fracture food properties. Compression tests are generally picked because they do not require a tight attachment between the sample and the testing fixture, simplifying sample preparation. Compression tests are limited by the maximum level of sample deformation. As a general rule, one should not use compression testing when normal engineering (Cauchy) strain exceeds 0.8 (i.e., 80% compression). Tension and torsion tests are used on very deformable foods and a high level of strain is needed to fracture the sample. Torsion requires a twisting force to deform a sample. Torsion testing is uniquely applicable to foods that lose water during testing. Water loss is prevented during torsional testing because the sample experiences true shear and does not change shape (5). The main disadvantage to tension and torsion tests is that the sample must be attached to the test fixture.

34.4.3.1.1 Determining Stress, Strain, and Elastic Modulus (E) in Compression

There are several assumptions to consider when doing compression testing. Along with the previously mentioned considerations of homogeneous and isotropic, the assumption that the food is an incompressible material greatly simplifies matters. An incompressible material is one that changes in shape but not volume when compressed. Foods such as frankfurters, cheese, cooked egg white, and other high-moisture gel-like foods generally are incompressible. The calculations for strain are as discussed previously (Equations [1] and [2]).

During compression, the initial cross sectional area (A_i) increases as the length decreases. To account for this change, a correction term incorporating a ratio of the cylinder lengths (L/L_i) is applied to the stress calculation.

$$\sigma = \frac{F}{A_i} \times (L/L_i) \qquad [30]$$

In compression testing one should use a cylindrical shaped sample with a length (L) to diameter ratio of > 1.0. The sample should be compressed between two flat plate with diameters exceeding the lateral expansion of the compressed sample (i.e., the sample should be in contact with the plates during testing). The equations are based on the sample maintaining a cylindrical shape when compressed. If this is not the case, the contact surface between the plate and the sample may need

lubrication. Water or oil can be used and one should pick the fluid that provides the desired lubrication without causing any deleterious effects to the sample.

A cylinder of cheddar cheese 3 cm in length (L_i), with an initial radius (R_i) of 1 cm, was compressed at a constant rate to 1.8 cm (L) and recorded a force of 15 N. Then:

$$\varepsilon_C = \frac{\Delta L}{L_i} = \frac{1.8 - 3.0}{3.0} = -0.4 = 0.4_{\text{compression}} \qquad [31]$$

$$\varepsilon_H = \ln(1 + \varepsilon_C) = \ln(1 + (-0.4))$$
$$= -0.5 = 0.5_{\text{compression}} \qquad [32]$$

$$A_i = \pi R_i^2 = 0.000314 \text{ m}^2 \qquad [33]$$

$$\sigma = \frac{F}{A_i} \times \left(\frac{L}{L_i}\right) = \frac{15 \text{N}}{0.000314 \text{ m}^2} \times \left(\frac{1.8}{3.0}\right)$$
$$= 28{,}700 \text{ Pa}_{\text{compression}} = 28.7 \text{ kPa}_{\text{compression}} \qquad [34]$$

$$E = \frac{\sigma}{\varepsilon_H} = \frac{28.7 \text{ kPa}}{0.5} = 57.4 \text{ kPa}_{\text{compression}} \qquad [35]$$

If the material compressed is a pure elastic solid, the compression rate does not matter. However, if the material is viscoelastic (as is the case with most foods), then the values for stress, strain, and elastic modulus will change with compression rate. A complete characterization of a viscoelastic material requires determining these values at a variety of compression rates. Another factor to consider is the level of compression. The sample can be compressed to fracture or some level below fracture. The goal should be compression to fracture if one wants to correlated rheological with sensory properties.

34.4.3.1.2 Texture Profile Analysis (TPA)

Texture profile analysis is an empirical technique using a two-cycle compression test, typically with a universal testing machine (Fig. 34-12). This test was developed by a group of food scientists from Kraft Foods and is compiled as force during compression and time. Data analyses correlated numerous sensory parameters, including hardness, cohesiveness, and springiness with texture terms determined from the TPA test curve. For example, the peak force required to fracture a specimen has been strongly related to sample hardness. Bourne (6) provides a more detailed description of TPA.

34.4.3.2 Small Strain Testing

The goal in small strain testing is to characterize the rheological properties of a material without damaging the material. This requires small forces and deformations. In addition, since most foods are viscoelastic, one needs to be able to separate viscous and elastic properties. These goals are accomplished by applying: (1) a stress or strain in oscillation and measuring the respec-

gain a more complete understanding of the rheological properties. The advantage of determining fundamental, rather than empirical, rheological properties is that it allows one to use common units, independent of the specific instrument, to determine the rheological property. This not only allows for comparison among values determined on different instruments, but it also permits a comparison of the flow of honey with the flow of paint. Through rheological methods, food scientists have the ability to relate theoretical and experimental information from a range of disciplines, including polymer chemistry and materials sciences, to gain a greater understanding of the quality and behavior of food materials.

34.6 GLOSSARY (INCLUDES TERMS IN SECTION 34.9)

Compression: A force acting in a perpendicular (normal) direction toward the body

Concentric Cylinder: A test fixture for rotational viscometry frequently called a cup and bob

Cone and Plate: A test fixture for rotational viscometry

Constitutive Equation: An equation relating stress with strain and sometimes other variables including time, temperature and concentration

Creep Test: The deformation of a material exposed to a constant stress

Deborah Number: The ratio of a characteristic time of a material to the characteristic time of the process

Dilatent: Shear-dependent thickening

Empirical Test: Simple tests measuring poorly defined parameters but typically found to correlate with textural or other characteristics

Fundamental Test: A measurement of well-defined, physically based rheological properties

Homogeneous: Well mixed and compositionally similar regardless of location

Incompressible: No change in material density

Isotropic: The material response is not a function of location or direction

Kinematic Viscosity: The viscosity divided by the density of the material

Laminar Flow: Nonturbulent flow

Linear Viscoelasticity: Viscoelasticity within the region where stress and strain are linearly related

Loss Modulus: The out-of-phase component of the shear stress divided by the strain during exposure to a sinusoidally varying input function

Modulus: A ratio of stress to strain

Newtonian Fluid: A fluid with a linear relationship between shear stress and shear rate without a yield stress

Non-Newtonian Fluid: Any fluid deviating from Newtonian behavior

34-12 figure Photo of universal testing machine.

tive strain or stress and phase angle between stress and strain, (2) a constant strain and measuring the decrease (relaxation) in stress, or (3) a constant stress and measuring the rate of deformation (creep). A more detailed description of these techniques may be found in section 34.9.

34.5 SUMMARY

Rheological testing is simple in that it only requires the measurement of force, deformation, and time. To convert these measurements into fundamental physics-based rheological properties requires an understanding of the material and testing method. Materials should be homogeneous and isotropic. This is true for most fluid foods and many solid foods. Fundamental rheological properties are determined based on knowledge of the stress or strain (normal or shear) applied to the sample and the geometry of the testing fixture. Once rheological properties are determined they can be described by physical or mathematical models to

No-Slip: The fluid velocity adjacent to a moving boundary has the same velocity as the boundary

Oscillatory Test: Dynamic test using a controlled sinusoidally varying input function of stress or strain

Pseudoplastic: Shear-thinning

Rheology: A science studying how all materials respond to applied stresses or strains

Rheogram: A graph showing rheological relationships

Rheometer: An instrument measuring rheological properties

Rheopectic: Time-dependent shear-thickening

Shear (Strain) Rate: Change in strain with respect to time

Simple Shear: The relative motion of a surface with respect to another parallel surface creating a shear field within the fluid contained between the surfaces

Simple Shear Approximation: A prediction technique for shear rate estimation of fluids within a narrow gap

Steady Shear: A flow field in which the velocity is constant at each location with time

Steady State: Independent of time

Storage Modulus: The in-phase component of the shear stress divided by the strain during exposure to a sinusoidally varying input function

Strain: Relative deformation

Stress: Force per unit area

Stress Relaxation Test: The decrease of stress within a material exposed to a constant strain

Tension: A force acting in a perpendicular direction away from the body

Test Fixture: A rheological attachment, sometimes called a geometry, that shears the sample material

Thixotropic: Time-dependent shear-thinning

Torque: A force generating rotation about an axis and is the product of the force and the perpendicular distance to the rotation axis

Torsion: A twisting force applied to a specimen

Viscoelastic: A material having both viscous and elastic properties

Viscometer: An instrument measuring viscosity

Viscosity: An internal resistance to flow

Yield Stress: A minimum stress required for flow to occur

NOMENCLATURE

Symbol	Name	Units
A	area	m^2
A_i	initial sample area	m^2
De	Deborah number	unitless
E	modulus of elasticity	Pa
E_e	equilibrium elasticity	Pa
E_o	initial elasticity	Pa
F	force	N

Symbol	Name	Units
G	shear modulus	Pa
G^*	shear complex modulus	Pa
G'	shear storage modulus	Pa
G''	shear loss modulus	Pa
h	height	m
J	creep compliance	Pa^{-1}
K	consistency coefficient	Pa sec^n
L	length	m
L_i	initial length	m
ΔL	change in length	m
M	torque	N m
n	flow behavior index	unitless
r	radial distance	m
R	radius	m
R_i	initial radius	m
R_b	bob radius	m
R_c	cup radius	m
t	time	sec
t	characteristic time of material	sec
U	velocity	m sec^{-1}
ε	normal strain	unitless
ε_C	engineering strain (Cauchy)	unitless
ε_H	true strain (Hencky)	unitless
γ	shear strain	unitless
γ	angle of shear	radians or degrees
γ_o	strain amplitude	unitless
$\dot{\gamma}$	shear (strain) rate	sec^{-1}
δ	phase angle	radians
η	apparent viscosity	Pa sec
η^*	complex viscosity	Pa sec
θ	angle	radians or degrees
θ	cone angle	radians or degrees
μ	Newtonian viscosity	Pa sec
μ_{pl}	plastic viscosity	Pa sec
σ	stress	Pa
σ_b	shear stress at the bob	Pa
σ_o	yield stress	Pa
σ_o	stress amplitude	Pa
ω	angular velocity	radians sec^{-1}
Ω	angular velocity	radians sec^{-1}

34.7 STUDY QUESTIONS

1. What is the difference between a shear and normal stress?
2. How is stress different from force?
3. What is the definition of apparent viscosity? When does the apparent viscosity equal the Newtonian viscosity?
4. Silly Putty[*] is a viscoelastic material.
   a. What is viscoelasticity?
   b. Explain the concept of Deborah Number.

c. What conditions will the Silly Putty[9] respond with a De << 1? With De >> 1?

5. Vegetable oil is Newtonian fluid and catsup is a Bingham Plastic fluid. What are the differences in flow behavior and how does it alter the food applications of these fluids?

6. Apple sauce at 26°C may be described by the following mathematical expression:

$$\sigma = 5.6 \gamma^{0.45}$$

$$\sigma = Pa$$
$$\gamma = 1/sec$$

a. Which rheological model is it? Identify the constants, K and n, with proper units.
   Honey at 26°C obeys a Newtonian model:

$$\sigma = 8.9 \gamma$$

Evaluate the apparent viscosity for apple sauce and honey at a shear rate of 1.0, 2.8, and 5.6 sec^{-1}.

b. How do the viscosities compare for each material? How do the viscosities compare at each shear rate?

c. Describe the importance of multipoint testing.

7. If you were designing a new "chip dip," what type of rheological considerations might you suggest?

8. What are the differences between empirical and fundamental rheological tests? Be creative and develop two new empirical tests, such as recording the amount of time required for spaghetti sauce to drain from a colander. Then, identify fundamental rheological tests that could be used to determine similar properties from your empirical tests. Explain advantages of using fundamental rheological tests.

ADVANCED QUESTIONS (SEE SECTION 34.9)

1. Using rheology, explain how to determine the gelation point temperature.
2. What is the phase angle for any Newtonian fluid? Why?

34.8 REFERENCES

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34.9 APPENDIX—ADVANCED RHEOLOGICAL METHODS

34.9.1 Oscillation—Controlled Stress or Strain Testing

There are a variety of commercial rheometers operating on the basic principle of applying a shear stress or strain in oscillation and measuring the respective torque or deformation and the phase relationship between viscous and elastic components. The fundamental principles can be explained based on the patterns shown for stress and strain in Fig. 34-13. Note that a sinusoidal oscillation has an amplitude (this is a maximum level of stress or strain) and a duration (time it takes to complete one oscillatory cycle). Duration of a single oscillatory cycle is called frequency (f in Hz or cycles/sec) or more precisely reported as radians/sec ($\omega = 2\pi f$, radians/sec). With these instruments the operator adjusts the amplitude and frequency of the input signal and measures the responding stress or strain value. Using the example of a controlled strain instrument, the strain input function is:

$$\gamma = \gamma_0 \sin(\omega t) \qquad [36]$$

where γ_0 is the strain amplitude shown in Fig. 34-13A. The resulting stress is:

$$\sigma = \sigma_0 \sin(\omega t - \delta) \qquad [37]$$

with δ being the phase angle.

The meaning of phase angle can be illustrated using Fig. 34-13A,B. In applying a sinusoidal strain, the test fixture rotates one direction to a set strain amplitude (γ_0), then reverses direction and goes to the same amplitude. At γ_0 the strain is a maximum and the strain rate is zero. This position is most sensitive to elastic properties. When the strain oscillation crosses the starting point (zero strain) it is at maximum shear rate or speed. Because viscosity is the relationship between shear stress and shear rate, this point is of maximum sensitivity to viscous behavior (elasticity is not detected because there is no strain on the sample). If the input and response sinusoidal curves superimpose along the time axis, the angle between the two curves (δ) is zero (Fig. 34-13A), reflecting ideal elastic behavior. Pure elastic solids have a phase angle of zero regardless of oscillation frequency, because stress is related to the magnitude of strain, not the strain rate. If the curves are shifted such that maximum stress occurs
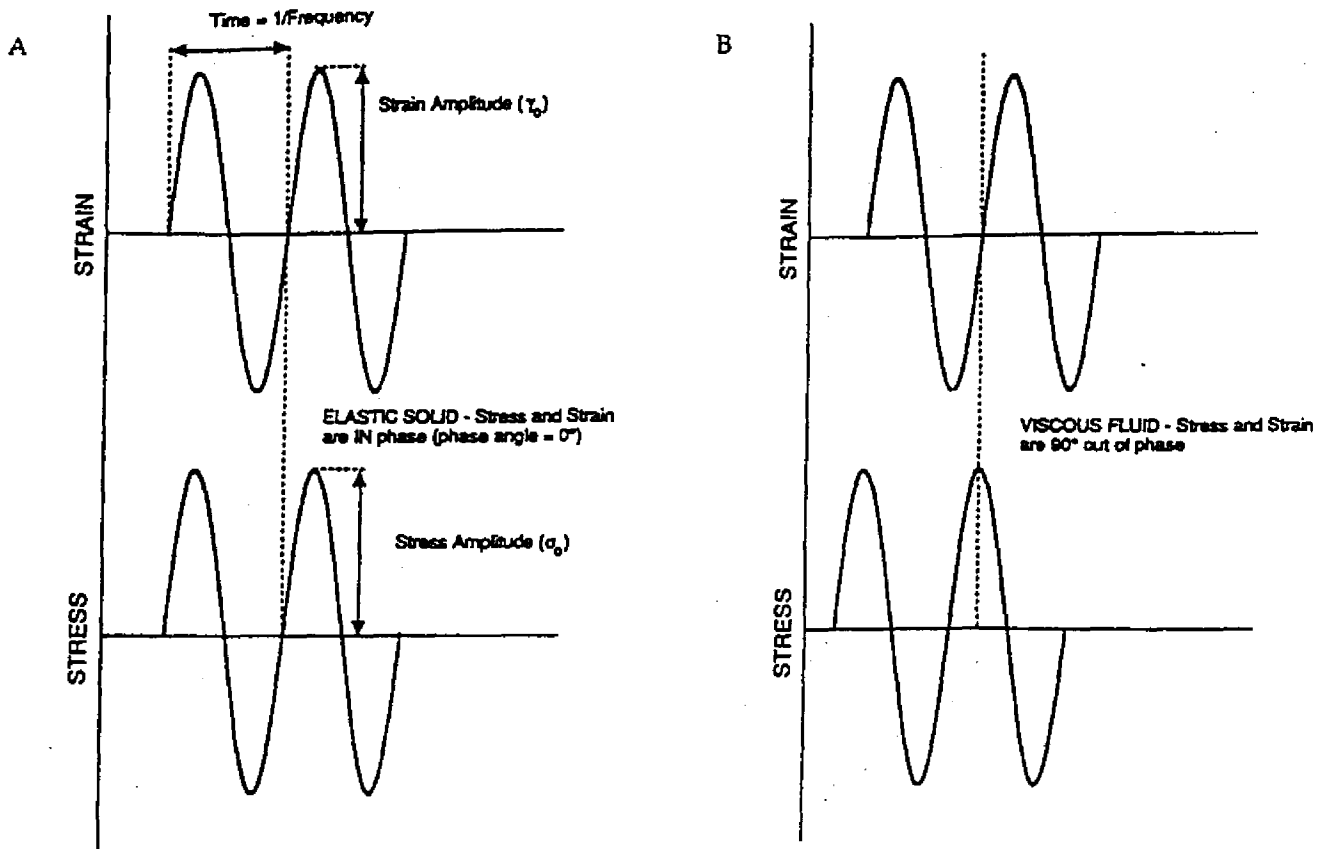
34-13
figure

Controlled rate instrument showing a response for an ideal (A) elastic and (B) viscous material.

at zero strain, then the phase angle is 90° (Fig. 34-13B), and the material is considered ideally viscous. Pure viscous fluids have a phase angle of 90° because that is the point of maximum strain rate.

Viscoelastic solids and fluids have a phase angle between 0 and 90°. A material with a phase angle approaching 90° will be dominated by viscous behavior; likewise, a sample with a phase angle closer to 0° will behave more elastically. The phase angle is not a fixed property; it will vary with the testing frequency. Recall the concept of the Deborah number. At faster oscillations, there will be less time for bigger molecules to relax or flow and the food will appear to be more solidlike (i.e., no detectable flow under the conditions of testing).

34.9.1.1 Oscillation Methods

Instruments are engineered so that the stress or strain may be controlled while measuring the responding torque or deformation response. In controlled rate instruments (controlled strain) a sinusoidal strain function is applied to the sample. The operator sets the

strain amplitude and frequency and the instrument measures the responding stress. With controlled stress instruments a sinusoidal stress function is applied and the resulting strain is measured. In all testing one sets the level of stress and strain to be at the maximum sensitivity without causing damage to the sample. The goal is to measure within the linear viscoelastic region. This is the range where stress and strain are linearly related and the moduli (G^*, G', and G'') are independent of stress or strain (Fig. 34-14). This region is established by running stress or strain sweeps. In each case, the respective parameter is increased and a modulus is determined. The range where the modulus is constant is the linear viscoelastic region.

The storage and loss moduli (G' and G'') are parameters representing the relative degrees of elastic and viscous behavior of viscoelastic materials. G' is the portion inphase, and G'' is the component 90° out-of-phase with the input strain. Therefore, a sample with a larger G' component will behave elastically (solidlike), while a material with a higher G'' will be more viscous (fluidlike). The following sections further develop these concepts.
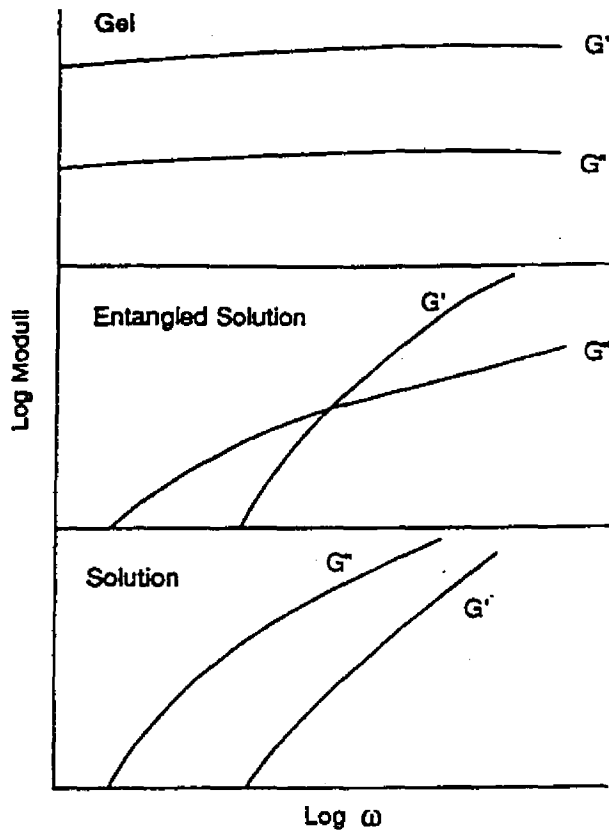
Figure 34-16 Frequency sweeps of three different viscoelastic materials.
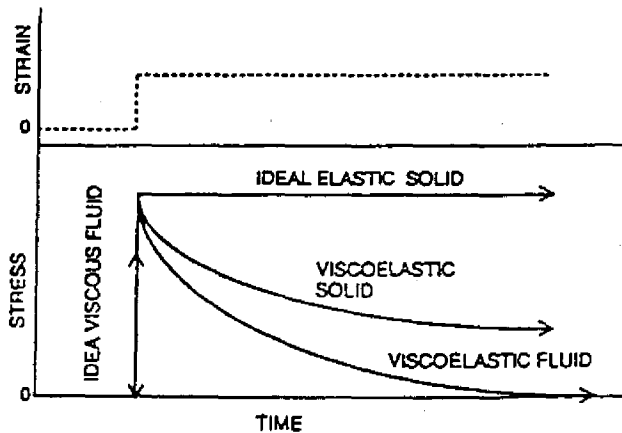
Figure 34-17 Concepts of stress relaxation testing.

ior, the stress will be constant and proportional to the strain. If the material has an ideal viscous behavior, then the stress will rapidly decay to zero as the material flows to adjust to the level of strain. Viscoelastic materials show a slow decay to zero for a viscoelastic fluid or an equilibrium elasticity for a viscoelastic solid. Various

approaches to analyzing stress relaxation data have been outlined by Nussinovitch et al. (7).

Let us assume that a strain is applied to a material in compression or extension and we record the decrease (relaxation) of stress. This means that the elastic modulus (stress–strain) is not a constant but a function of time. This is represented as $E(t)$. A modified Maxwell model can be used to represent stress relaxation data.

$$\frac{\sigma(t)}{\gamma_{constant}} = E(t) = E_e + (E_o - E_e)\exp\left(\frac{-t}{\tau}\right) \qquad [38]$$

With viscoelastic solids, the stress will eventually stop decreasing at the point where the remaining stress represents the equilibrium elasticity (E_e). With viscoelastic fluids, $E_e = 0$. In practice it may be difficult to establish a true E_e because the material will continue to relax for hours, days, and even weeks! An alternative is to use an extrapolation procedure to obtain E_e (8) or to pick a reasonable time for an "apparent" E_e.

With a modified Maxwell model the material can be described by three constant factors: initial modulus (E_o) which is the first reading at maximum strain, equilibrium modulus (E_e), and relaxation time (τ). The relaxation time constant is the time it takes for the stress to decay to $1/e$ or 36.8% of its initial value (Fig. 34-18). Since $1/e = 0.368$, the relaxation time is found by determining the time corresponding to $E = E_e + (E_o - E_e) 0.368$. Once these factor are determined, one can check how well the model represents the data by comparing the model with the experimental data. If the fit is poor, one should consider more complex models.

34.9.3 Creep—Constant Stress Testing

Creep tests are like stress relaxation tests but a constant stress is applied rather than a constant strain. The simplest creep test would be to apply a weight on top of a
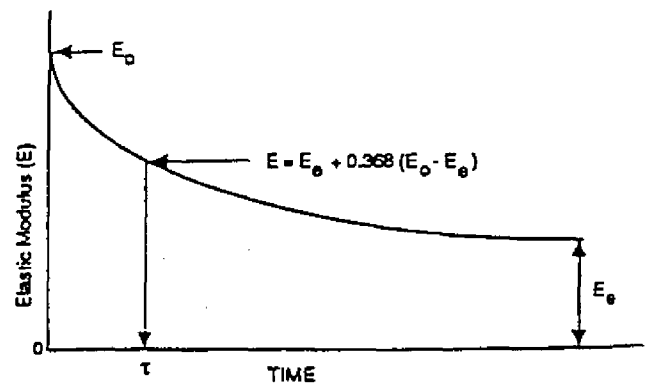
Figure 34-18 Decay of elastic modulus.

sample and record the change in shape (strain) over time, for example, placing a book on a cake and measuring the deformation over time. Conditions should be set to minimize sample damage and maximize sensitivity. Since deformation is a function of time, the test duration is controlled in creep testing to minimize sample damage. The general principles are seen in Fig. 34-19. Stress is applied and held constant. With elastic materials the level of strain coincides with the level of stress and remains constant until the stress is removed, then returns to zero. Viscous materials flow and the strain increases with time. There is no recovery once the stress is removed and the strain remains at the highest level. Viscoelastic materials show a combination of the two extremes.

Creep data is reported as a compliance modulus, which is stress/strain. The compliance function for a creep test is:

$$J(t) = \frac{\gamma(t)}{\sigma_{constant}} \qquad [39]$$

Note the unit for creep compliance is 1/Pa. Controlled stress rheometers are generally used to conduct creep tests. There are various approaches but they all require
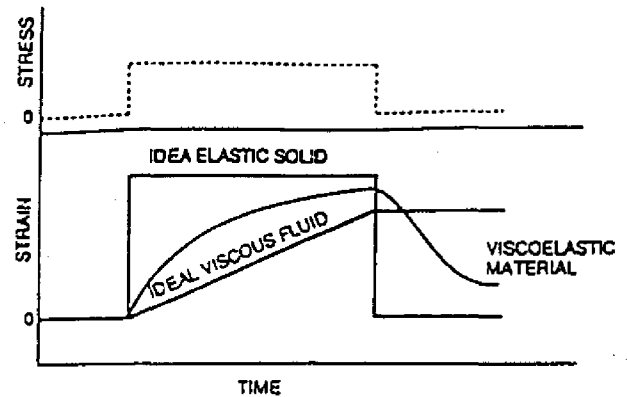
34-19
figure

Concepts of creep testing.

a preset frequency, stress, and maximum strain. A typical experiment would involve applying a stress for a period of time then having a strain recovery period which is call a creep recovery test. The creep recovery test should be as long as the creep test. The degree of strain recovered is an indication of the elasticity of the material.

Analysis of Food Emulsions

D. Julian McClements

35.1 INTRODUCTION

35.1.1 Importance of Emulsions in Foods

A great many food products exist either partly or wholly as emulsions, or have been in an emulsified state sometime during their production, including milk, cream, mayonnaise, salad dressings, butter, margarine, low fat spreads, sauces, soups, coffee whiteners, and cream liquors (1–3). The appearance, texture, and stability of these products depends on their composition, microstructure, and colloidal interactions (1–4). Improvement of food quality, reduction of manufacturing costs, development of new food products, and optimization of food processing operations depend on the availability of analytical techniques to characterize the properties of food emulsions. Analytical techniques are needed for research and development to identify the major factors that govern the properties of food emulsions, and in quality assurance laboratories to monitor the properties of foods during processing. The overall composition of food emulsions can be analyzed using many of the procedures described elsewhere in this book. Similarly, their rheological properties can be analyzed using the methods described in Chapter 34. In this chapter, we concentrate on those properties that are unique to emulsions, namely disperse phase volume fraction, droplet size distribution, droplet charge, physical state of droplets, emulsion stability, and emulsifier efficiency.

35.1.2 Classifications and Definitions

An emulsion consists of two immiscible liquids (oil and water), with one of the liquids dispersed as small spherical droplets in the other (Fig. 35-1). In foods, the

Stable Emulsion / Phase inversion

Creaming   Flocculation   Coalescence
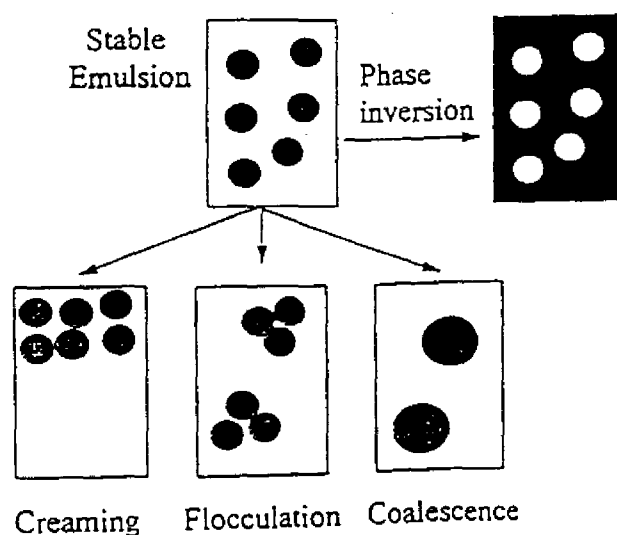
35-1
figure
Major mechanisms of emulsion instability.

diameter of these droplets typically ranges between 0.1 and 100 μm. A system that consists of oil droplets dispersed in an aqueous phase is called an oil-in-water (or O/W) emulsion, e.g., mayonnaise, milk, cream, soups, and sauces. A system that consists of water droplets dispersed in an oil phase is called a water-in-oil (or W/O) emulsion, e.g., margarine, butter, and spreads. The substance within the droplets is refered to as the dispersed or internal phase, whereas the substance that makes up the surrounding liquid is called the continuous or external phase. It is also possible to create multiple emulsions, which can be of either the oil-in-water-in-oil type (O/W/O) or the water-in-oil-in-water type (W/O/W). The process of converting bulk oil and bulk water into an emulsion, or of reducing the size of the droplets in an existing emulsion, is known as homogenization. In the food industry, homogenization is usually achieved by applying intense mechanical agitation to a liquid using a mechanical device known as a homogenizer, e.g., a high pressure valve homogenizer, an ultrasonic homogenizer, or a colloid mill (1–4).

Emulsions are thermodynamically unstable systems because the contact between oil and water molecules is energetically unfavorable, and so they tend to breakdown with time. The preparation of emulsions that are kinetically stable over a time period that is of practical use to the food industry (e.g., a few days, weeks, months, or years) requires the incorporation of substances known as emulsifiers and/or thickening agents. An emulsifier is a surface-active molecule that adsorbs to the surface of an emulsion droplet to form a protective coating that prevents the droplets from aggregating with one another, e.g., an amphiphilic protein, a phospholipid, or a small molecule surfactant. An emulsifier also reduces the interfacial tension and therefore facilitates the disruption of emulsion droplets during homogenization. A thickening agent is a substance that increases the viscosity of the continuous phase and therefore slows down the movement of the droplets, e.g., many polysaccharides.

35.1.3 Emulsion Properties

The concentration of droplets in an emulsion is usually described by the dispersed phase volume fraction, ϕ, which is equal to the volume of emulsion droplets (V_D) divided by the total volume of emulsion (V_E): $\phi = V_D/V_E$. In some situations, it is more convenient to express the composition of an emulsion in terms of the dispersed phase mass fraction (ϕ_m), which is related to ϕ by the following equation:

$$\phi_m = \phi\left(\phi + (1-\phi)\frac{\rho_1}{\rho_2}\right)^{-1} \quad [1]$$

where:

ϕ_m = dispersed phase mass fraction
ϕ = dispersed phase volume fraction
ρ_1 = continuous phase density
ρ_2 = dispersed phase density

When the densities of the two phases are equal, the mass fraction is equivalent to the volume fraction.

Many of the most important quality attributes of emulsion-based food products are governed by the size of the droplets they contain, and so it is important for food scientists to be able to control, predict, and measure droplet size. All food emulsions contain droplets that have a range of different sizes, i.e., they are polydisperse. Ideally, one would like to know the full particle size distribution, although in many situations it is only necessary to have an indication of the average droplet size and the width of the distribution. The most useful numbers for describing a particle size distribution are the mean diameter $\bar{d}$ and the standard deviation σ:

$$\bar{d} = \Sigma n_i d_i / N \qquad [2]$$

$$\sigma = \sqrt{(\Sigma n_i (d_i - \bar{d})^2)/N} \qquad [3]$$

where:

n_i = number of droplets with diameter d_i
N = total number of droplets

The above mean is often referred to as the mean length diameter, d_L, because it represents the sum of the *length* of the droplets divided by the total number of droplets. It is also possible to express the mean droplet size in a number of other ways to reflect different aspects of an emulsion, e.g., average surface area or average volume (5, 6). A particle size distribution is usually continuous, but it can be conveniently presented as a histogram (Fig. 35-2) or as a table (Table 35-1) by dividing the distribution into a number of size classes and recording the percentage of droplets in each size class. It should be stressed that the shape of a particle size distribution and the value of the mean diameter depends strongly on the way in which the concentration of droplets in each size group is represented: a plot of droplet number versus diameter has a different shape to a plot of droplet volume versus diameter. It is important to appreciate this fact because analytical instruments that measure particle size distributions report them in various ways.

The droplets in many food emulsions are prevented from aggregating by use of ionic emulsifiers that adsorb to their surface and prevent them from coming close together because of electrostatic repulsion. The electrical charge on a droplet is characterized by its zeta potential (ζ). If the pH or ionic strength of an emulsion is altered so that the electrostatic repulsion is

Diameter, d_i / μm
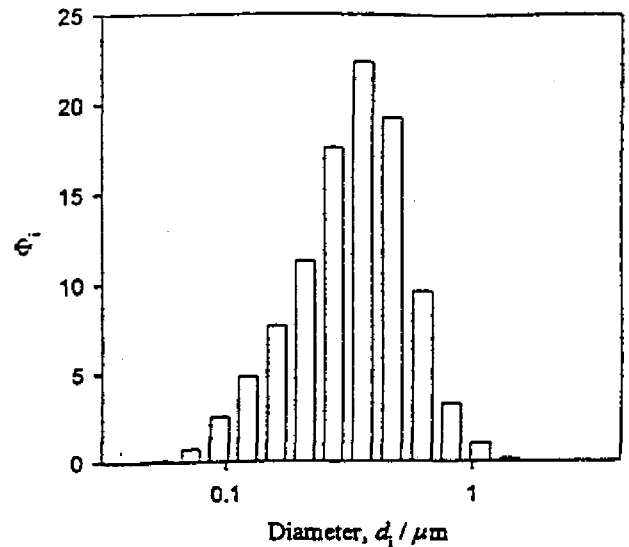
35-2   Histogram of particle size distribution of a typical emulsion. ϕ_i = volume frequency, d_i = droplet diameter.

35-1   Particle Size Distribution of an Emulsion Represented in Tabular Form

Size Class (μm)	Midpoint, d_i (μm)	Φ_i (%)[1]	Droplets with $d < d_i$ (%)[2]
0.041–0.054	0.048	0.00	0.00
0.054–0.071	0.063	0.12	0.00
0.071–0.094	0.082	0.71	0.12
0.094–0.123	0.108	2.48	0.83
0.123–0.161	0.142	4.79	3.31
0.161–0.211	0.186	7.62	8.10
0.211–0.277	0.244	11.2	15.70
0.277–0.354	0.320	17.56	26.92
0.354–0.477	0.420	22.33	44.48
0.477–0.626	0.551	19.76	66.81
0.626–0.821	0.723	9.52	86.07
0.821–1.077	0.949	3.21	95.59
1.077–1.414	1.245	1.03	98.81
1.414–1.855	1.634	0.17	99.85
1.855–2.433	2.144	0.00	100.00

[1]Φ_i is the volume percentage of droplets in the stipulated size class.
[2]The cummulative size distribution represents the percentage of droplets below a certain size.

reduced below some critical level, the droplets aggregate. The tendency for droplet aggregation to occur in many oil-in-water emulsions is governed by their physical state due to partial coalescence (see next section). Extensive droplet aggregation causes a dramatic increase in emulsion viscosity, and may even lead to complete emulsion breakdown. Consequently, it is important for food analysts to have experimental techniques to quantify the electrical charge and physical

state of emulsion droplets, and to determine the degree of droplet aggregation.

35.1.4 Emulsion Stability

There are a number of physicochemical mechanisms responsible for the breakdown of food emulsions, the most important being creaming/sedimentation, flocculation, coalescence, partial coalescence, and phase inversion (Fig. 35-1). Creaming is the process in which droplets move upwards because of gravity because they have a lower density than the surrounding liquid. Sedimentation is the process in which droplets move downwards due to gravity because they have a higher density than the surrounding liquid. Flocculation is the process in which two or more droplets "stick" together to form an aggregate in which the droplets retain their individual integrity. Coalescence is the process in which two or more droplets merge together to form a single larger droplet. Partial coalescence is the process in which two or more partly crystalline droplets merge together to form a single irregularly shaped aggregate due to the penetration of a solid fat crystal from one droplet into a region of liquid oil in another droplet. Phase inversion is the process in which an oil-in-water emulsion changes to a water-in-oil emulsion, or vice versa. Partial coalescence and phase inversion are an integral part of many food processing operations, such as the production of butter, margarine, and whipped cream. Generally, the term emulsion stability refers to its ability to resist changes in its physicochemical properties with time. Nevertheless, it is always important to be clear about which mechanism is responsible for the instability.

35.2 TESTING EMULSIFIER EFFICIENCY

One of the most important decisions that a food manufacturer must make when developing an emulsion-based food product is the selection of the most appropriate emulsifier. A huge number of emulsifiers are available as ingredients in foods, and each has its own unique properties and optimum range of applications. The efficiency of an emulsifier is governed by a number of its characteristics, including the minimum amount required to produce a stable emulsion; its ability to prevent droplets from aggregating over time; the speed at which it adsorbs to the droplet surface during homogenization; and the interfacial tension, thickness, and viscoelasticity of the interfacial membrane. These characteristics depend on the nature of the food in which the emulsifier is present, e.g., pH, ionic strength, ion type, ingredient interactions, temperature, and mechanical agitation. It is therefore important to test emulsifier efficiency under conditions that are similar

to those found in the actual food product in which it is going to be used (7). A number of procedures commonly used to test emulsifier efficiency are discussed below.

35.2.1 Emulsifying Capacity

It is often important for a food manufacturer to know the minimum amount of an emulsifier that can be used to create a stable emulsion. The emulsifying capacity of a water-soluble emulsifier is defined as the maximum amount of oil that can be dispersed in an aqueous solution containing a specific amount of the emulsifier without the emulsion breaking down or inverting to a water-in-oil emulsion (7). Experimentally, it is determined by placing an aqueous emulsifier solution into a vessel and continuously agitating using a high-speed blender as small volumes of oil are titrated into the vessel. The endpoint of the titration occurs when the emulsion breaks down or inverts, which can be determined by optical, viscosity, or electrical conductivity measurements. The greater the volume of oil that can be incorporated into an emulsion before it breaks down, the higher the emulsifying capacity. Although this test is widely used to characterize emulsifiers, it has a number of drawbacks that limit its application as a standard procedure. The results of the test depend on the type of blender or homogenizer used, the rate at which the oil is titrated into the vessel, the method used to determine the endpoint, the initial concentration of emulsifier used, and the temperature at which the test is carried out. The emulsifying capacity therefore should be regarded as a qualitative index, which depends on the specific conditions used to carry out the test.

A more reliable means of characterizing the minimum amount of emulsifier required to form an emulsion is to measure the surface load (Γ_s), which corresponds to the mass of emulsifier required to cover a unit area of droplet surface. A stable emulsion is prepared by homogenizing known amounts of oil, water, and emulsifier. The mass of emulsifier adsorbed to the surface of the droplets per unit volume of emulsion ($C_{adsorbed}$/kg m^{-3}) is equal to the initial emulsifier concentration minus that remaining in the aqueous phase after homogenization (which is determined by centrifuging the emulsion to remove the droplets and then analyzing the serum). The total droplet surface area covered by the adsorbed emulsifier (S) is given by (5):

$$S = 6\varphi V_e / d \qquad [4]$$

where:

φ = dispersed phase volume fraction
V_e = emulsion volume

d = volume-surface mean droplet diameter

Thus the surface load can be calculated (typically a few mg m^{-2}):

$$\Gamma_S = C_{adsorbed} V_e / S \qquad [5]$$

A knowledge of the surface load enables one to calculate the minimum amount of emulsifier required to prepare an emulsion containing droplets of a given size. In practice, excess emulsifier is needed because it does not all adsorb to the surface of the droplets during homogenization.

35.2.2 Emulsion Stability Index

An efficient emulsifier produces an emulsion in which there is no visible separation of the oil and water phases with time. Phase separation may not become visible to the human eye for a long time, even though some emulsion breakdown has occurred. Consequently, it is important to have analytical tests that can be used to detect the initial stages of emulsion breakdown, so that their long-term stability can be predicted. One widely used test is to centrifuge an emulsion at a given speed and time and observe the amount of creaming and/or oil separation that occurs (7). This test can be used to predict the stability of an emulsion to creaming using relatively low centrifuge speeds, or to coalescence by using speeds that are high enough to rupture the interfacial membranes. The greater the degree of creaming or oil separation that occurs, the greater is the instability of an emulsion, and the less efficient is the emulsifier. Although this test is widely used and can be carried out fairly rapidly, it does have a number of important limitations: the rate of creaming or coalescence in a centrifugal field may not be a good indication of emulsion instability under normal storage conditions, and it does not take into account chemical or biochemical reactions that might alter emulsion stability over extended periods.

A more quantitative method of determining emulsifier efficiency is to measure the change in the particle size distribution of an emulsion with time using one of the analytical techniques discussed in section 35.3. An efficient emulsifier produces emulsions in which the particle size distribution does not change over time, whereas a poor emulsifier produces emulsions in which the particle size increases due to coalescence and/or flocculation. The kinetics of emulsion stability can be established by measuring the rate at which the particle size increases with time. These tests should be carried out under similar conditions as found in the final product, e.g., pH, ionic strength, composition, temperature, etc.

35.2.3 Surface and Interfacial Tension

One of the most valuable means of obtaining information about the characteristics of an emulsifier is to measure the reduction in surface tension when it adsorbs to an interface. Surface tension measurements can be used to determine the kinetics of emulsifier adsorption, the packing of emulsifier molecules at an interface, critical micelle concentrations, surface pressures, and competitive adsorption (1–4). Surface tension (γ_s) is defined as the amount of energy (ΔE) required to increase the surface area between a fluid and a gas (e.g., air and water) by an amount ΔA:

$$\Delta E = \gamma_s \Delta A \qquad [6]$$

By analogy, the interfacial tension (γ_i) is defined as the amount of energy required to increase the interfacial area between two immisicble fluids (e.g., oil and water). The origin of surface and interfacial tensions is the imbalance of molecular interactions across an interface (5, 6). For example, molecular interactions between oil and water are thermodynamically unfavorable because of the hydrophobic effect, and so energy must be supplied to increase the contact area between these molecules. The origin of the hydrophobic effect is the fact that the introduction of a nonpolar molecule into water causes alterations in the interactions and structural organization of the water that is energetically unfavorable (5).

Emulsifiers reduce the tension at an interface by "shielding" the unfavorable molecular interactions across it. The dependence of the surface tension on bulk emulsifier concentration for a typical nonionic surfactant is illustrated in Fig. 35-3. Initially, the surface tension decreases linearly with the logarithm of emulsifier concentration (x), by an amount that depends on the excess surface concentration (Γ) of the emulsifier:

$$\Gamma = -\frac{1}{RT} \left(\frac{\delta \gamma_s}{\delta \ln(x)} \right) \qquad [7]$$

where:

R = gas constant
T = absolute temperature
γ_s = surface tension
x = emulsifier concentration
δ = indicates a differential

The excess surface concentration is equal to the amount of emulsifier at the surface (over and above that which would be present if the molecule were not surface active) divided by the surface area, and is thus related to the surface load mentioned in the previous section. Γ is determined from the slope of the initial part of a plot of surface tension versus log concentra-

d = volume-surface mean droplet diameter

Thus the surface load can be calculated (typically a few mg m^{-2}):

$$\Gamma_S = C_{adsorbed}V_e/S \qquad [5]$$

A knowledge of the surface load enables one to calculate the minimum amount of emulsifier required to prepare an emulsion containing droplets of a given size. In practice, excess emulsifier is needed because it does not all adsorb to the surface of the droplets during homogenization.

35.2.2 Emulsion Stability Index

An efficient emulsifier produces an emulsion in which there is no visible separation of the oil and water phases with time. Phase separation may not become visible to the human eye for a long time, even though some emulsion breakdown has occurred. Consequently, it is important to have analytical tests that can be used to detect the initial stages of emulsion breakdown, so that their long-term stability can be predicted. One widely used test is to centrifuge an emulsion at a given speed and time and observe the amount of creaming and/or oil separation that occurs (7). This test can be used to predict the stability of an emulsion to creaming using relatively low centrifuge speeds, or to coalescence by using speeds that are high enough to rupture the interfacial membranes. The greater the degree of creaming or oil separation that occurs, the greater is the instability of an emulsion, and the less efficient is the emulsifier. Although this test is widely used and can be carried out fairly rapidly, it does have a number of important limitations: the rate of creaming or coalescence in a centrifugal field may not be a good indication of emulsion instability under normal storage conditions, and it does not take into account chemical or biochemical reactions that might alter emulsion stability over extended periods.

A more quantitative method of determining emulsifier efficiency is to measure the change in the particle size distribution of an emulsion with time using one of the analytical techniques discussed in section 35.3. An efficient emulsifier produces emulsions in which the particle size distribution does not change over time, whereas a poor emulsifier produces emulsions in which the particle size increases due to coalescence and/or flocculation. The kinetics of emulsion stability can be established by measuring the rate at which the particle size increases with time. These tests should be carried out under similar conditions as found in the final product, e.g., pH, ionic strength, composition, temperature, etc.

35.2.3 Surface and Interfacial Tension

One of the most valuable means of obtaining information about the characteristics of an emulsifier is to measure the reduction in surface tension when it adsorbs to an interface. Surface tension measurements can be used to determine the kinetics of emulsifier adsorption, the packing of emulsifier molecules at an interface, critical micelle concentrations, surface pressures, and competitive adsorption (1–4). Surface tension (γ_s) is defined as the amount of energy (ΔE) required to increase the surface area between a fluid and a gas (e.g., air and water) by an amount ΔA:

$$\Delta E = \gamma_s \Delta A \qquad [6]$$

By analogy, the interfacial tension (γ_i) is defined as the amount of energy required to increase the interfacial area between two immisicble fluids (e.g., oil and water). The origin of surface and interfacial tensions is the imbalance of molecular interactions across an interface (5, 6). For example, molecular interactions between oil and water are thermodynamically unfavorable because of the hydrophobic effect, and so energy must be supplied to increase the contact area between these molecules. The origin of the hydrophobic effect is the fact that the introduction of a nonpolar molecule into water causes alterations in the interactions and structural organization of the water that is energetically unfavorable (5).

Emulsifiers reduce the tension at an interface by "shielding" the unfavorable molecular interactions across it. The dependence of the surface tension on bulk emulsifier concentration for a typical nonionic surfactant is illustrated in Fig. 35-3. Initially, the surface tension decreases linearly with the logarithm of emulsifier concentration (x), by an amount that depends on the excess surface concentration (Γ) of the emulsifier:

$$\Gamma = -\frac{1}{RT}\left(\frac{\delta \gamma_s}{\delta \ln(x)}\right) \qquad [7]$$

where:

R = gas constant
T = absolute temperature
γ_s = surface tension
x = emulsifier concentration
δ = indicates a differential

The excess surface concentration is equal to the amount of emulsifier at the surface (over and above that which would be present if the molecule were not surface active) divided by the surface area, and is thus related to the surface load mentioned in the previous section. Γ is determined from the slope of the initial part of a plot of surface tension versus log concentra-

Surface
Tension
γ / mN

CMC

π

Surfactant Concentration

Increasing Concentration

Below
CMC

Above
CMC

35-3
figure

Measurements of the dependence of surface tension on bulk emulsifier concentration provide valuable information about an emulsifier. CMC = critical micelle concentration, π = surface pressure.

tion (Fig. 35-3). Above a certain bulk surfactant concentration, the surface becomes saturated with emulsifier molecules and the surface tension tends to a constant value. This concentration corresponds to the critical micelle concentration (CMC) of the surfactant: below this concentration the surfactant molecules exist predominantly as monomers, whereas above it they form micelles (Fig. 35-3). The decrease of surface tension below the original value (when no emulsifier is adsorbed) is known as the surface pressure, Π. When a surface becomes saturated with emulsifier, there is a maximum surface pressure (Π_{max}) that can be achieved, which depends on the emulsifier type. This value governs the stability of emulsion droplets to disruption when they are mechanically agitated: the higher Π_{max}, the less stable the droplets are to disruption. Measurements of the dependence of the surface tension on bulk emulsifier concentration can therefore provide information about Γ, Π_{max}, and CMC.

Surface and interfacial tensions are measured

using analytical instruments called surface or interfacial tensiometers. Different types of tensiometer are available that vary according to the design of the instrument; whether surface tension, interfacial tension or both can be measured; and whether measurements are static or dynamic. Static measurements are carried out on surfaces or interfaces that are at equilibrium, whereas dynamic measurements are carried out on systems that are not at equilibrium. Dynamic measurements are particularly useful for monitoring the kinetics of emulsifier adsorption. A number of the most important types of tensiometer are listed in Table 35-2. The Wilhelmy plate method is one of the most widely used in the food industry, and only it will be considered here. Descriptions of the other types of tensiometer can be found elsewhere (S).

The liquid to be analyzed is placed in a thermostated beaker (Fig. 35-4). A thin platinum–irridium plate, which is attached to a sensitive force measuring device, is positioned so that the bottom edge of the

35-2 **table**

Summary of Instruments Used to Measure Surface and Interfacial Tensions

Surface Tension	Interfacial Tension
Equilibrium	
Du Nouy ring	Du Nouy ring
Wilhelmy plate	Wilhelmy plate
Pendant drop	Pendant drop
Sessile drop	Sessile drop
Spinning drop	Spinning drop
Capillary rise	
Dynamic	
Maximum bubble pressue	Drop volume
Oscillating jet	Pendant drop
Drop volume	Spinning drop
Surface waves	

35-4 **figure**
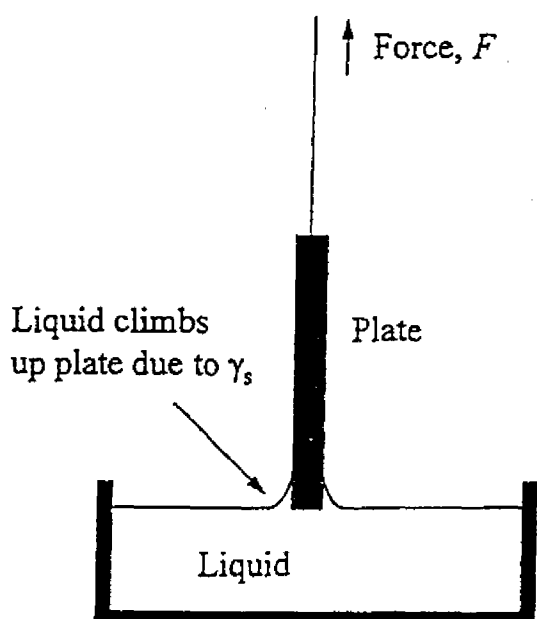
Wilhelmy plate tensionmeter for measuring the surface tension of liquids. γ_s = surface tension.

plate is tangential to the surface of the liquid. A small amount of liquid climbs up the side of the plate because of its surface tension, and exerts a downward pull on the plate because of its weight. This weight is recorded by the force measuring device and is related to the surface tension by:

$$\gamma_s = W/P \cos\theta \qquad [8]$$

where:

W = plate weight
P = plate perimeter
θ = contact angle between liquid and plate

The same technique can be used to measure interfacial tensions, but in this case the plate is positioned at the interface between the oil and water phases, and a correction has to be made for the bouyancy of the plate.

35.2.4 Interfacial Rheology

The rate of droplet coalescence in many emulsions depends on the rheological properties of the interfacial membrane that surrounds the droplets. Interfacial rheology is the two-dimensional equivalent of bulk rheology (Chapter 34), and consequently many of the principles and concepts are directly analogous. An interface can be viscous, elastic, or viscoelastic depending on the type, concentration, and interactions of the molecules present. Two types of deformation are particularly important at an interface: shear and dilitation (9). The shear behavior characterizes the resistance to the movement of interfacial regions past one another, without there being any change in the interfacial area. Dilatation refers to the resistance to the expansion or contraction of the interfacial area. Interfacial shear behavior is often characterized using an instrument analogous to the concentric cylinder technique used to analyze bulk materials (Chapter 34). The sample is placed in a thermostatted dish, and a thin disk is placed in the plane of the interface that separates the two phases (Fig. 35-5). The dish is rotated and the torque on the disk is measured. Interfacial dilatational behavior can be determined by measuring the change in surface tension as the interfacial area is increased in a controlled manner (10).

35.3 DROPLET SIZE DISTRIBUTION

35.3.1 Microscopy

A number of microscopic techniques can be used to determine the particle size distribution of emulsions

35-5 **figure**
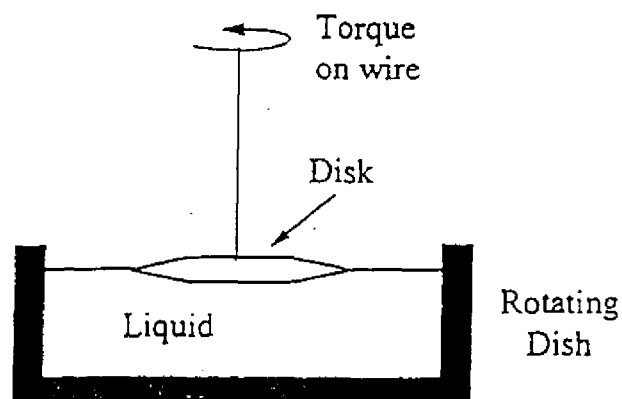
Device for measuring interfacial shear behavior.

(11, 12). Optical microscopy utilizes a series of lenses to direct light through a specimen and to magnify the resulting image. The resolution of an optical microscope is about 0.5 μm, which means that it is only suitable for analyzing emulsions with fairly large droplets. Emulsions containing smaller droplets can be analyzed using scanning electron microscopy (resolution ≈4 nm) or transmission electron microscopy (resolution ≈0.4 nm). Electron microscopes use electron beams, rather than light beams, to generate an image of an emulsion. Microscopy offers the most direct means of determining the particle size distribution of an emulsion, although it does have a number of limitations. The preparation of samples is often time-consuming and laborious, and may damage any delicate structures within the emulsion, e.g., flocs. In addition, a number of different regions within a sample must be analyzed to obtain statistically satisfactory data.

35.3.2 Static Light Scattering

Static light scattering techniques are used to determine the size distribution of particles with diameters between about 0.1 and 1000 μm, and therefore are suitable for analyzing most food emulsions (5, 13). A dilute emulsion is placed in a glass cuvette and a laser beam is directed through it (Fig. 35-6). The laser beam is scattered by the droplets by an amount that depends on

their size, which leads to the formation of a characteristic angular dependence of the intensity of the light emerging from the emulsion, known as a scattering pattern. The scattering pattern is detected by an array of photodiodes located behind the glass cuvette. A computer program calculates the particle size distribution which gives the best-fit between the measured scattering pattern and that predicted by light scattering theory. Most light scattering instruments present the data in the form of a table (Table 35-1) or histogram (Fig. 35-2). Commercial instruments are fully automated, simple to use, and provide an analysis of an emulsion within a few minutes. The major disadvantage of this technique is that emulsions must be transparent and contain low concentrations of droplets ($\phi < 0.1$ wt %) so that multiple scattering effects are negligible. Most food emulsions therefore must be diluted considerably prior to analysis, which means the technique is destructive, cannot be used directly for on-line measurements, and may cause disruption of flocculated droplets.

The mean droplet size of an emulsion can also be determined by spectroturbidimetric methods. The emulsion to be analyzed is placed in a cuvette and its turbidity is measured over a range of wavelengths (typically between 300 and 800 nm). The droplet size is then determined by finding the best-fit between the experimental measurements of turbidity versus wavelength,

Dilute Emulsion   Detector
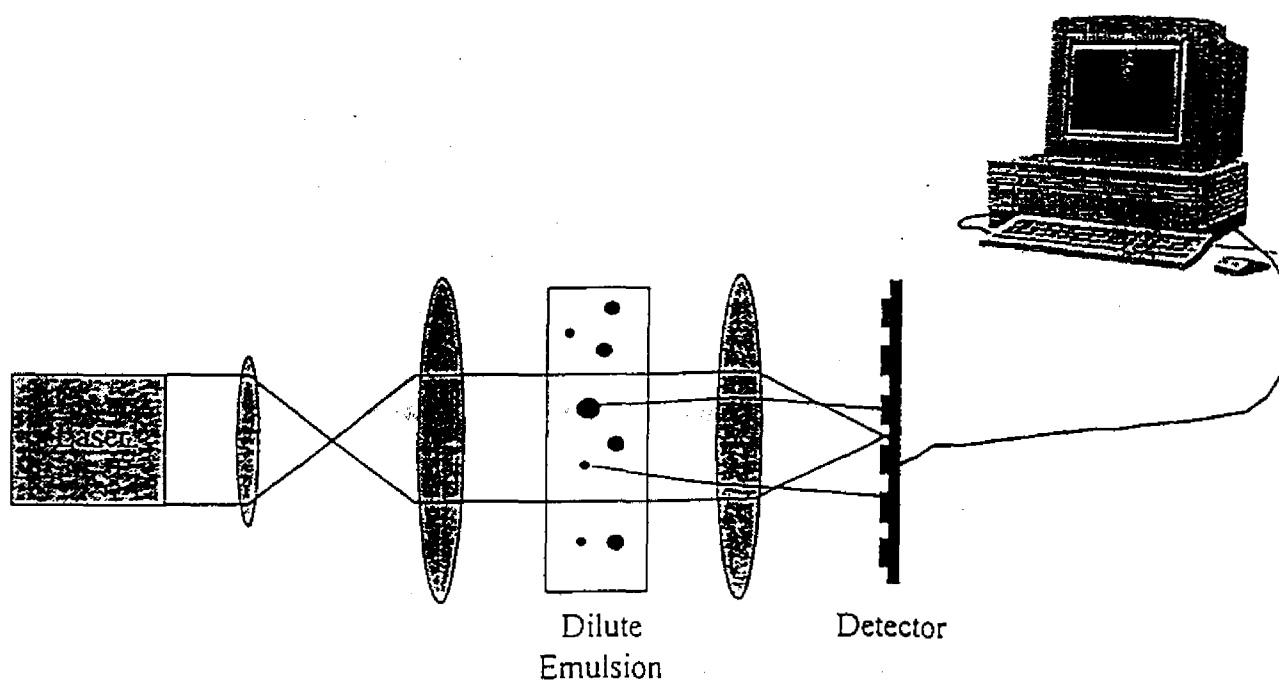
35-6 Static light scattering technique for determining droplet size distributions of emulsions.
figure

and those predicted by light scattering theory. The spectroturbimetric technique can be carried out using the UV-visible spectrophotometers found in most research laboratories, which may circumnavigate the need to purchase one of the expensive commercial light scattering instruments mentioned previously.

35.3.3 Dynamic Light Scattering

Dynamic light scattering techniques are used to determine the size distribution of particles with diameters between about 3 nm and 3 μm, and therefore are unsuitable for analysis of many food emulsions, because they contain larger droplets (5, 13). Even so, it is useful for determining the size of protein aggregates, surfactant micelles, and small emulsion droplets. When a laser beam is reflected from a moving droplet its frequency is shifted by an amount that depends on the droplet velocity: the faster the droplet moves, the greater the frequency shift. The droplets in an emulsion are in continual motion because of their thermal energy, with the smaller droplets moving more rapidly than the larger ones. Thus the particle size distribution can be determined by measuring the frequency shift of a laser beam reflected from an emulsion. A number of instruments are available that utilize this principle, including Photon Correlation Spectroscopy and Doppler Shift Spectroscopy (5, 6). A number of these dynamic light scattering techniques are capable of analyzing emulsions that are fairly concentrated (ϕ < 30%). Commercial instruments are available that are easy to use and that can analyze an emulsion in a few minutes. One of the major limitations of this technique is that the viscosity of the aqueous phase must be Newtonian, which may not be the case for many food emulsions, especially those that contain thickening agents.

35.3.4 Electrical Pulse Counting

Electrical pulse counting techniques are capable of determining the size distribution of particles with diameters between about 0.4 and 400 μm, and therefore are suitable for analyzing most food emulsions. The sample to be analyzed is placed in a beaker that has two electrodes dipping into it (Fig. 35-7). One of the electrodes is contained within a glass tube that has a small hole in it, through which the emulsion is sucked. When an oil droplet passes through the hole it causes a decrease in the current between the electrodes because oil has a significantly lower electrical conductivity than water. Each time a droplet passes through the hole, the instrument records a decrease in current that it converts into an electrical pulse. The instrument controls the volume of liquid that passes through the hole and so the droplet concentration can be determined by counting the number of electrical pulses in a known
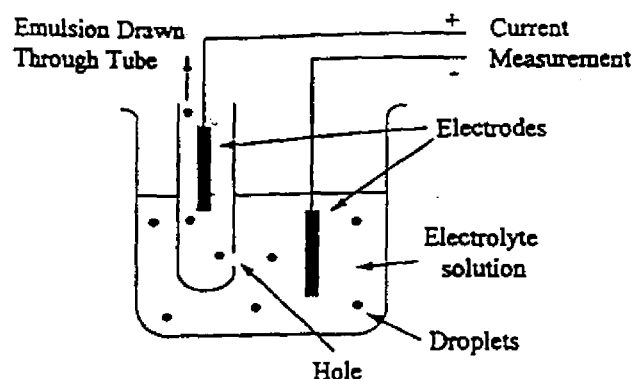
Electrical pulse counting device for measuring particle size distributions.

volume. The pulse height increases as the size of the droplets increases, and so the particle size distribution can be determined by measuring the height of each pulse as the droplets pass through the hole (once a calibration has been carried out using a standard of known particle size). Instruments are easy to use and can analyze an emulsion sample within a few minutes. The major limitations of the technique are that emulsions must be diluted so that only one droplet passes through a hole at a time, and the droplets must be suspended in an electrolyte solution to increase the magnitude of the current between the electrodes. Dilution of an emulsion in an electrolyte solution can promote flocculation or alter the structure of existing flocs and therefore cause misleading results.

35.3.5 Sedimentation

Sedimentation methods can be used to determine the size distribution of particles between about 1 nm and 1 mm, although a number of different instruments have to be used to cover the whole of this range. Particle size is determined by measuring the velocity at which droplets sediment (or cream) in a gravitational or centrifugal field. The velocity (v) that an isolated rigid spherical particle suspended in an ideal liquid moves due to gravity is given by Stokes equation:

$$v = \frac{2(\rho_2 - \rho_1)gr^2}{9\eta_1}$$ [9]

where:

ρ_2 = disperse phase density
ρ_1 = continuous phase density
η_1 = continuous phase viscosity
g = acceleration due to gravity
r = droplet radius

The movement of the droplets can be monitored using a variety of experimental methods, including visual observation, optical microscopy, ultramicroscopy, light scattering, nuclear magnetic resonance (NMR), ultrasound, and electrical measurements. This approach cannot be used to analyze droplets with diameters smaller than a few micrometers because the movement of these droplets due to gravity is opposed by the disorganizing influence of the thermal energy. This problem can be overcome by applying a centrifugal force to the emulsion to increase the rate at which the droplets move through the liquid.

When an emulsion is placed in a centrifuge and rotated rapidly, it is subjected to a centrifugal force which causes droplets that have a lower density than the surrounding liquid to move inwards (i.e., oil-in-water emulsions), and droplets that have a higher density to move outwards (i.e., water-in-oil emulsions). The droplet velocity $v(x)$ depends on the angular velocity of the rotor, ω, and their distance from the center of the rotor, x. It is convenient to characterize the motion of the droplets by a sedimentation coefficient (S), which is independent of rotation speed and droplet position:

$$S = \frac{v(x)}{\omega^2 x} \qquad [10]$$

The sedimentation coefficient is determined by measuring the position of the droplets in the centrifuge tube with time, which can be carried out using a variety of methods, including visual observation, X-ray adsorption, and light scattering (5). The radius (r) of an isolated spherical particle in a fluid is related to the sedimentation coefficient by the following equation:

$$r = \sqrt{\frac{9\eta_1 S}{2(\rho_2 - \rho_1)}} \qquad [11]$$

where:

η_1 = continuous phase velocity
S = sedimentation coefficient
ρ_2 = disperse phase density
ρ_1 = continuous phase density

Commercial instruments are capable of measuring the full particle size distribution of emulsions by analyzing the velocity of a large number of droplets. This technique is capable of highly accurate measurements; nevertheless, it is often time-consuming to carry out, can only be used to analyze dilute emulsions, and requires that the droplets be suspended in a Newtonian liquid.

35.3.6 Emerging Technologies

Most of the traditional methods of determining particle size distributions require that the emulsion be prepared in some way prior to analysis. Recently, a number of

analytical techniques have been developed that can be used to measure the particle size distribution of emulsions that are concentrated and optically opaque without the need of any form of sample preparation, e.g., ultrasonics, electroacoustics, dielectrics, and NMR. Particle sizing instruments based on ultrasonic and electroacoustic spectroscopy are commercially available for analyzing concentrated emulsions. Ultrasonic spectroscopy relies on the fact that the scattering of high-frequency sound waves from an emulsion depends on the size of the droplets it contains (14), whereas electroacoustic spectroscopy relies on the fact that the movement of charged particles in an electric field depends on their size (5). NMR (15) and dielectric spectroscopy (16) techniques have been developed to analyze concentrated emulsions, but commercial instruments are not yet available. Analytical instruments based on these emerging technologies will be particularly suitable for on-line determination of the particle size distribution of food emulsions.

35.4 DISPERSE PHASE VOLUME FRACTION

The concentration of droplets in an emulsion can be determined using many of the standard analytical methods covered elsewhere in this book, e.g., the oil content can be determined by solvent extraction or nonsolvent extraction techniques (Chapter 13) or the water content can be determined by evaporation, distillation, or chemical techniques (Chapter 8). Many of these techniques are labor intensive, time consuming, and destructive, and so are unsuitable for rapid quality assurance tests. One of the simplest nondestructive methods of determining the disperse phase volume fraction (ϕ) of an emulsion is to measure its density $(\rho_{emulsion})$, and the density of the continuous (ρ_1) and dispersed (ρ_2) phases:

$$\phi = \frac{\rho_{emulsion} - \rho_1}{\rho_2 - \rho_1} \qquad [12]$$

The density of an emulsion can be measured using a variety of different experimental techniques, including density bottles, hydrometers, and oscillating U-tube devices (17).

The disperse phase volume fraction also can be measured using many of the techniques used to determine droplet size distributions. Static light scattering and electrical pulse counting techniques can be used to determine disperse phase volume fractions in dilute emulsions ($\phi < 0.1\%$), whereas dynamic light scattering, ultrasonic, electroacoustic, dielectric, and NMR techniques can be used to analyze much more concentrated emulsions. All of these techniques rely on there being a measureable change in some physicochemical property of an emulsion as its droplet concentration

increases, e.g., the intensity of scattered light, the attenuation of an ultrasonic wave, the amplitude or decay time of an NMR signal, or the electrical conductivity of an emulsion.

35.5 DROPLET CRYSTALLINITY

In food emulsions, we are concerned mainly with the crystallization of fat droplets in oil-in-water emulsions, rather than water droplets in water-in-oil emulsions. This is because the crystallization of fat droplets is more likely to occur at food temperatures and because it can cause dramatic changes in the stability and texture of emulsions due to partial coalescence. The physical state of emulsified fats can be monitored using experimental techniques that utilize differences in the physicochemical properties of the solid and liquid phases (e.g., density, compressibility, electrical conductivity, molecular mobility, or packing), or changes associated with the solid-liquid phase transition (e.g., heat absorption/release). The physical properties that are of particular interest to the food scientist are: the final melting point of the fat; the fraction of fat that is crystalline at a particular temperature; the morphology, interactions, and location of the crystals; and the packing of the molecules within the crystals (18).

The solid fat content (SFC) is the fraction of a fat that is crystalline at a particular temperature. The variation of the SFC with temperature can be measured conveniently using a variety of techniques, including density measurements, differential scanning calorimetry, dielectric measurements, nuclear magnetic resonance, ultrasonic velocity measurements, and electron spin resonance (18). The technique used in a particular experiment depends on the equipment available, the information required, and the nature of the sample being analyzed. Information about the location of crystals within an emulsion droplet and their morphology can be studied by polarized light microscopy or by electron microscopy depending on the size of the crystals.

The packing of the molecules in the crystals can be determined by techniques that utilize the scattering or adsorption of radiation. X-ray diffraction and small angle neutron scattering have been used to determine the long and short spacings of the molecules in fat crystals. Infrared and Raman spectroscopy have been used to obtain information about molecular packing via its effect on the vibration of certain chemical groups in fat molecules. Each polymorphic form has a unique spectrum that can be used to identify it. The polymorphic form of fat crystals also can be identified by measuring the temperature at which phase transitions occur and the amount of heat absorbed/released using differential scanning calorimetry (DSC) or differential thermal analysis (DTA) (Chapter 36).

35.6 DROPLET CHARGE

35.6.1 Electrophoresis

The emulsion to be analyzed is placed in a measurement cell and a static electric field E is applied across it via a pair of electrodes, which causes any charged emulsion droplets to move toward the oppositely charged electrode (Fig. 35-8). The sign of the droplet charge is deduced from the direction they move. When an electric field is applied across an emulsion the droplets accelerate until they reach a constant velocity (v), where the electrical pulling force is exactly balanced by·the viscous drag force exerted by the surrounding liquid. This velocity (v) depends on droplet charge, and therefore can be used to provide information about the zeta potential (ξ):

$$\xi = \frac{\eta v}{E \varepsilon_0 \varepsilon_R} \qquad [13]$$

where:

η = viscosity of surrounding liquid
E = static electric field
ε_0 = dielectric constant of a vacuum
ε_R = relative dielectric constant of surrounding liquid

This equation is suitable for fairly large droplets, and in general a more complex expression is needed to interpret the data (5, 6). Particle velocity is determined experimentally by measuring the distance they move in a known time, or the time taken to move a known distance. The motion of the particles can be monitored using a number of methods, including optical microscopy and light scattering (5, 6).

35.6.2 Laser Interference Electrophoresis

A more sophisticated way of measuring the zeta potential of emulsion droplets is to use laser interference electrophoresis. Two coherent beams of light are made to intersect at a particular position within a measurement cell so that they form an interference pattern consisting of regions of low and high light intensity (5, 6). The charged emulsion droplets are made to move through the interference pattern by applying an electric field across the cell. As the droplets move across the interference pattern they scatter light in the bright regions, but not in the dark regions. The faster a droplet moves through the interference pattern, the greater the frequency of the intensity fluctuations. By analyzing the frequency of these fluctuations it is possible to determine the particle velocity, which can then be related to the zeta potential (e.g., using Equation [9]). The sign of the charge on the particles is ascertained
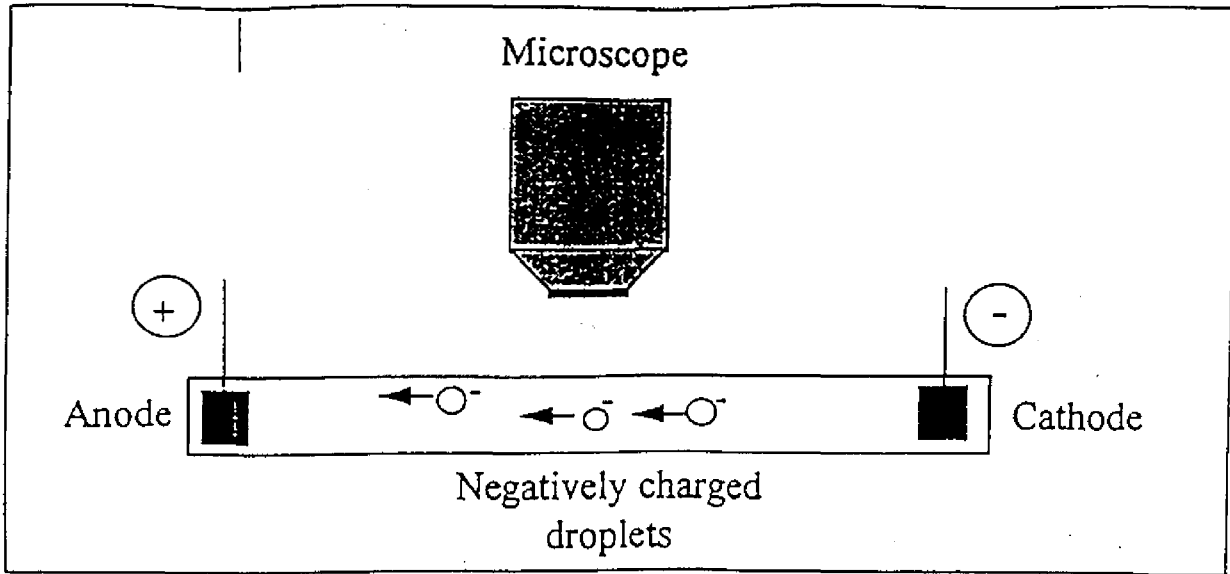
Electrophoretic mobility cell for measuring the zeta potential of emulsion droplets.

from the direction they move in the electric field. Commercial instruments based on this principle also are capable of measuring droplet size distributions (from 3 nm to 3 μm) using a dynamic light scattering technique. Consequently, it is possible to determine the droplet size and charge using a single instrument, which is extremely valuable for predicting the stability and bulk physicochemical properties of food emulsions.

35.6.3 Electroacoustics

Recently, analytical instruments based on electroacoustics have become commercially available for measuring the zeta potential of emulsion droplets (5, 6). The sample to be analyzed is placed in a measurement cell and an alternating electrical field is applied across it via a pair of electrodes. This causes any charged droplets to move backwards and forwards in response to the alternating electric field (Fig. 35-9). An oscillating droplet generates a pressure wave, with the same frequency as the electric field, which is detected by an ultrasonic transducer. The amplitude of the pressure wave received by the transducer is known as the electrokinetic sonic amplitude (ESA) and is proportional to the dynamic mobility, μ_d, of the droplets, which is related to their zeta potential and size:

$$\mu_d = \left(\frac{\varepsilon \cdot \varepsilon_R \zeta}{\eta}\right) G\left(\frac{\omega r^2 \rho}{\eta}\right) \qquad [14]$$

where:

ε_0 = dielectric constant of a vacuum
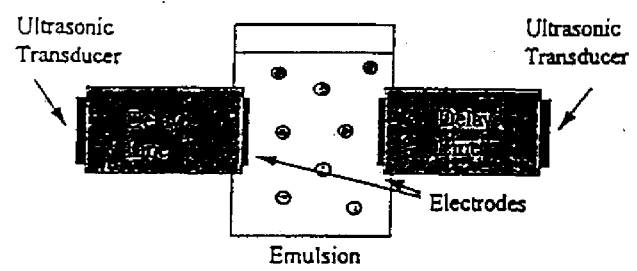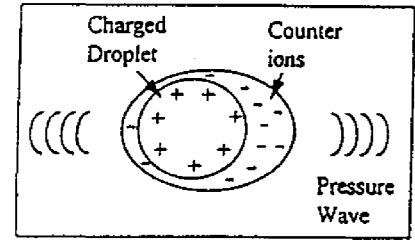
Electroacoustic technique for measuring the zeta potential and size distribution of charged emulsion droplets.

ε_R = relative dielectric constant of surrounding liquid

ζ = zeta potential

ω = angular frequency of alternating electrical field

r = droplet radius

η = continuous phase viscosity

ρ = continuous phase density

G = a function that varies from 1 at low frequencies to 0 at high frequencies

The frequency dependence of G is associated with the phase lag between the alternating electrical field and the oscillating pressure wave generated by the particles because of the particle inertia. At low frequencies, the particles move in-phase with the electric field, and so the dynamic mobility is equal to the static mobility (i.e., $G = 1$):

$$\mu_d = \varepsilon_0 \varepsilon_R \zeta / \eta \qquad [15]$$

At extremely high frequencies, the electric field alternates so quickly that the particles have no time to respond and therefore remain stationary. Under these circumstances no ultrasonic pressure wave is generated by the particles (i.e., $G = 0$). The transition from the low- to high-frequency regions occurs at a characteristic relaxation frequency ω_R that is related to the size of the particles, decreasing as the size (inertia) of the particles increases. The droplet size distribution therefore can be determined by measuring the frequency dependence of the dynamic mobility, whereas the zeta potential is determined by measuring the dynamic mobility at low frequencies (where it is independent of droplet size). Thus it is possible to determine both the size and zeta potential of droplets using a single instrument. The major advantage of the electroacoustic technique is that it can be applied to concentrated emulsions ($\phi \leq$ 30%) without the need for sample dilution. Nevertheless, it can be used only to analyze emulsions that contain droplets that are charged and that have a significant density difference to the surrounding liquid.

35.7 EMULSION STABILITY

35.7.1 Creaming/Sedimentation

The simplest method of determining the stability of an emulsion to creaming is to store it for a given time and then measure the height of the interface between the opaque creamed layer (containing droplets) and the transparent serum layer (devoid of droplets) using a ruler. The main limitations of this technique are: (1) it may be necessary to wait for a long time before creaming is observable, (2) the serum layer may not be transparent, and (3) it does not give any details of the distribution of droplets within the creamed layer. The creaming rate can be enhanced using accelerated creaming tests. The emulsion is placed in a transparent tube and centrifuged at a fixed speed for a given time, and then the height of the creamed layer is measured using a ruler. A number of analytical techniques have been developed to provide details of the spatial distribution of droplets in emulsions. Dilute emulsions can be analyzed by pouring an emulsion into a tall transparent test tube and measuring the transmittance and reflection of a light beam directed at it as a function of sample height. The creaming profile of concentrated

emulsions can be determined by making ultrasonic, NMR, or dielectric measurements as a function of sample height.

35.7.2 Droplet Aggregation

Droplet aggregation occurs due to a variety of physicochemical processes that lead to flocculation, coalescence, and partial coalescence (section 35.1.4). The extent of droplet aggregation can be ascertained by measuring the change in the particle size distribution of an emulsion with time using one of the analytical techniques mentioned in section 35.3. One of the most important facts to establish is whether the increase in droplet size is due to flocculation or coalscence. The most direct method of distinguishing between flocculation and coalescence it to observe the emulsion using optical or electron microscopy. In concentrated emulsions it may be difficult to ascertain whether droplets are flocculated or just in close contact. In addition, sample preparation may promote flocculation or alter floc structure and therefore cause misleading results. The degree of flocculation of protein-stabilized emulsions often can be established by adding a water-soluble surfactant (such as 1% Tween 20) to the aqueous phase. The surfactant is more surface-active than the protein and displaces it from the interface, causing any flocs to be disrupted. The particle size distribution is measured before and after the addition of the surfactant. If the droplets were flocculated there is a decrease in droplet size, but if they were coalesced the size remains the same. If there is extensive crosslinking of the proteins at the droplet interface due to disulfide bond formation, it may be necessary to add mercaptoethanol with the surfactant. A more indirect method of distinguishing between flocculation and coalescence is to measure the rheology of an emulsion, there being a dramatic increase in the viscosity of an emulsion when the droplets flocculate, but little change when they coalesce.

35.7.3 Phase Inversion

The conversion of an emulsion from an oil-in-water to a water-in-oil state can be monitored by a number of different techniques. Electrical measurements are commonly used for this purpose because there is a dramatic decrease in electrical conductivity when an oil-in-water emulsion inverts to a water-in-oil emulsion. Phase inversion can also be monitored by measuring the change in the viscosity of an emulsion.

35.8 SUMMARY

The most important properties of emulsion-based food products are the emulsifier efficiency, droplet size distribution, disperse phase volume fraction, zeta poten-

tial, and physical state. These colloidal properties largely determine bulk physicochemical properties of foods, such as appearance, texture, and stability. A wide variety of analytical techniques are available to characterize the properties of food emulsions. Many of the most important of these techniques have been reviewed in this chapter. The efficiency of an emulsifier can be established by either empirical or quantitative tests. Droplet size distributions can be determined by optical microscopy, static and dynamic light scattering, electrical pulse counting, and sedimentation techniques. Disperse phase volume fraction can be determined by many of the same techniques, as well as by density measurements. Droplet crystallinity can be determined by dilatometry, differential thermal analysis, differential scanning calorimetry, NMR, and ultrasonics. The electrical charge on emulsion droplets can be determined by electrophoresis or electroacoustics. The technique chosen for a particular application depends on the equipment available, the nature of the emulsion, and the cost of purchasing and running the equipment. It should be noted that there are a large number of additional analytical techniques that are used often in research and development laboratories and empirical tests that are used in industry that could not be covered in this chapter.

35.9 STUDY QUESTIONS

1. You are working for a large food company that is developing a new emulsion-based food product. The company finds that an oil-rich layer is forming on top of the product after a few days. Which experimental techniques would you use to try to identify and solve this problem?

2. Describe how differential scanning calorimetry (Chapter 36) could be used to monitor the crystallization of fat droplets in an oil-in-water emulsion, and the type of thermograms you might expect to see when the emulsion was cooled and heated.

3. What are the advantages of using a density measurement, rather than solvent extraction, to determine the disperse phase volume fraction of an emulsion?

4. Discuss the advantages and disadvantages of the different methods that can be used to determine the droplet size distribution of emulsions.

5. How would you distinguish between flocculation and coalescence of droplets in an oil-in-water emulsion stabilized by a milk protein?

6. a. What is emulsion stability?
   b. Describe the major physicochemical processes that cause emulsion instability.

7. What experimental tests would you use to determine which emulsifying ingredient would be most suitable for a particular food product?

8. Calculate the minimum droplet size that can be obtained by homogenizing 10 g of oil, 89 g of water, and 1 g of emulsifier, assuming that the emulsifier has a surface load of 2 mg m^{-2} and the oil has a density of 900 kg m^{-3}.

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36
chapter

Thermal Analysis

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36.1 INTRODUCTION

Thermal analysis is a broad term that encompasses numerous techniques that measure chemical or physical changes of a substance as a sample is subjected to a controlled temperature program over time. Food products or food components are chemically complex mixtures. They may include small molecules such as sugars with dextro or levorotatory forms, and the chemical properties, solubilities, and reactivities can be quite different in their different forms. Sometimes the molecular constituents can form different phases in water at different water contents and temperatures. An example would be the micellar states of surfactants. Other constituents are natural polymers, such as amylose and amylopectin from starch, or actin and myosin from muscle tissue.

The ultimate chemical change that can be monitored in a food system by thermal analysis is the total combustion of a product in order to determine its mineral and caloric contents. This is usually determined by bomb calorimetry and is achieved by uncontrolled heating of the material in an O_2 atmosphere once this material is ignited.

Over a desired temperature range, various methods of thermal analysis can measure:

1. Temperatures of transition (temperature when a phase change or chemical reaction begins, reaches a peak, or ends)
2. Heat capacity changes
3. Weight losses or gains
4. Energies of transition or enthalpic changes (ΔH)
5. Dimensional or volumetric changes
6. Viscoelastic or mechanical property changes during phase changes or reactions
7. Changes in electrical polarization
8. Evolved gases

The most popular modern thermal analysis techniques are those that dynamically follow (a sequence of) physicochemical changes that a substance undergoes during heating or cooling. Differential scanning calorimetry (DSC), differential thermal analysis (DTA), and thermogravimetric analysis (TGA) are three common dynamic thermoanalytical techniques. More recently, thermomechanical analysis (TMA) and dynamic mechanical thermal analysis (DMTA) have been developed as well. There are specialized forms of DSC, such as polarization DSC, that are considered to be beyond the scope of this chapter and are mentioned to alert the reader to their existence.

TGA measures changes in the weight of a sample as a function of temperature; both losses and gains can occur. TMA measures changes in penetration (softness/hardness), extension, expansion, or contraction

of a sample as a function of temperature. DMTA measures the mechanical properties of a substance, e.g., the viscous or elastic moduli. These properties are affected by polymer chain movement at a fixed or changing temperature. With proper instrumentation, the technique can be very sensitive. Such measurements are important in studies where maintaining or losing rigidity is important.

DTA and DSC have been the most commonly used methods of thermal analysis in food science. They measure the differential temperature or heat flow to or from a sample versus a reference material, and this is displayed as a function of temperature or time. These techniques can differentiate between two types of thermal events: endothermic (taking up heat) and exothermic (giving off heat). Because DSC and DTA are used very commonly in thermal analysis of foods, the remainder of this chapter will focus mainly on these techniques (especially DSC).

36.2 PRINCIPLES AND PROCEDURES

36.2.1 General Principle of Calorimetry

Calorimetry involves the measurement of temperature or heat, more specifically, the determination of the temperature and/or the quantity of heat absorbed or given off when a definite amount of material undergoes a specific chemical or physicochemical change. Chemical changes in foods are indicated by the following energy transformations:

1. Oxidation is accompanied by a net amount of heat being released during a temperature rise in the sample. These are called exothermic reactions.
2. Hydrolysis is characterized by little or no heat evolution. Reactions are almost isothermic.
3. Reduction is accompanied by a net amount of heat absorbed during a temperature rise. Reactions are endothermic (energy is taken up by the sample).

Physicochemical changes give rise to thermal transitions that can be summarized as:

1. Endothermic events that are caused by physical rather than chemical changes
   a. An endotherm over a narrow temperature range is indicative of crystalline rearrangement, crystalline melting (heat of fusion), or solid state transitions for pure materials.
   b. A broad transition is indicative of events that relate to dehydration, temperature-dependent phase behavior, or polymer melt.
2. Exothermic transitions that relate to processes without decomposition can be caused by a

decrease in enthalpy of a phase or chemical system.

a. Narrow exotherms can result from crystallization (ordering or freezing) of a metastable system, whether undercooled organic, inorganic, amorphous polymer or liquid, or annealing of stored energy resulting from mechanical stress.

b. Broad exotherms can result from solid–solid phase transitions, chemical reactions, polymerization, or curing of polymers.

3. Exothermic events that relate to decomposition can be narrow or broad, depending on kinetic behavior.

4. Step changes in the heat flow of a material that can be seen simply as a small change in heat capacity (change in the baseline) with no well-defined peak being produced. This behavior is characteristic of glass transitions.

The first three types of transitions often are called first order, while the last is called second order.

The net amount of heat absorbed or released by a sample can be measured quantitatively in a calorimeter. Units of thermal energy include:

$$1\ cal/g = 1.8\ Btu/lb \qquad [1]$$
$$= 0.001\ kcal\ (Cal)/g$$
$$= 4.186\ J/g$$

Temperature is measured in °F, °C, or K:

$$T(°C) = [T(°F) - 32] \times 5/9; \qquad [2]$$
$$T(K) = T(°C) + 273.16$$

Heat flow is represented in either calories/sec or watts:

$$1\ watt = 1\ joule/sec \qquad [3]$$
$$= 0.2388\ calorie/sec$$

36.2.2 Dynamic Thermal Analyzers

Dynamic thermal analyzers are special calorimeters (e.g., DSCs, DTAs), in which a test sample and an inert material are used. Both test and reference samples are heated or cooled at the same time under identical conditions. The temperature of the test sample will be either higher or lower than that of the reference, depending on whether the reaction or change is net exothermic or endothermic, respectively. If no difference in temperature exists between test sample and reference, then an isothermal state exists. Even when a nearly isothermal state exists, a small temperature differential between test sample and reference may indicate slight differences in heat capacity and thermal conductivity or differences in the weight and density of the two materials. Thus, dynamic thermal analysis

can monitor a property change over a temperature range. Such changes can include phase transitions, molecular conformational changes, interactions with other constituents, or pyrolytic degradation.

As mentioned earlier, until recently the most common dynamic thermal analyzers used in food science were differential analyzers such as DSCs and DTAs. Therefore, it is important to explain the differences between them, as well as the type of data each one can deliver.

Standardization of DSC and DTA nomenclature began in 1965 with the International Confederation for Thermal Analysis (ICTA). ICTA also developed a set of standards with well characterized melting temperatures, such as indium, melts, and heat capacities, such as sapphire crystals. These were used to calibrate thermal analysis equipment, and the United States National Bureau of Standards (now called the National Institute of Standards and Technology) began marketing these standards in 1971. ICTA has defined DTA as "a technique for recording the difference in temperature between a substance and reference material against either time or temperature as the two specimens are subjected to identical temperature regimes in an environment heated or cooled at a controlled rate (1)." The data are obtained as a thermogram with an ordinate axis in temperature difference (ΔT) between sample and reference materials. The abscissa is time or temperature. A downward peak is an endotherm, while an upward peak is an exotherm. Athough this is the ICTA's recommendation to record data, one often sees the thermograms inverted. Therefore, care must be taken to determine whether a downward peak is endothermic or exothermic.

The amount of heat or energy that is absorbed or evolved during a physical or chemical change can be calculated from the area between the curve and an appropriate baseline. In DTA, because there may be slight differences in heat capacity and thermal conductivity between sample and reference as they are heated, the resulting thermograms may show slight changes in ΔT, even though there is not a physical or chemical change occurring. Therefore, the DTA cannot be used to obtain heat capacity data directly. Sometimes, a calibration constant can be used as a function of temperature with the use of computerized transformations to achieve such information.

The ICTA definition of DSC is "a technique for recording the energy necessary to establish a zero temperature difference between a substance and a reference material against either time or temperature, as the two specimens are subjected to identical temperature regimes in an environment heated or cooled at a controlled rate (1)." Based on this definition, one can see that the sample and the reference cells must have separate heating elements and temperature sensors. To maintain the same temperature between sample and

reference, the resistance of the temperature sensor must be changed to influence the rate by which heat is supplied to the sample and reference, respectively. The DSC curve represents, on the ordinate axis, the rate of energy absorption (heat flow) by the sample relative to the reference. Like for DTA, the abscissa is time or temperature. The heat capacity of the sample will algebraically add to the rate of the heat flow for the endothermic or exothermic process being monitored.

In Fig. 36-1 are found the schematics of the temperature sensors and resistance heat sources in the sample and reference areas of calorimeters (2). The first DTA was made by Robert-Austen in 1899. It took about 50 years to design a practical DTA and determine that a DTA peak gave a direct measure of the heat required to affect a physicochemical change in a material (3).

The general design of the classical DTA can be found in Fig. 36-1a, in which the thermocouples are embedded in the sample and a single heat source is used. Boersma (4) modified the classical DTA. The differences can be seen in Fig. 36-1b, where the thermocouples are embedded in the blocks containing the sample and reference cells, but still having one heater.

In 1964, Perkin-Elmer introduced the first commercial DSC (5). The sample and reference areas can be seen schematically in Fig. 36-1c. This technique involves recording the difference in energy flux necessary to establish a zero temperature difference between a substance and reference material against either time or temperature when both are heated and cooled. The heating or cooling takes place at a controlled rate. Separate heating and temperature sensing devices are used to attain this (usually temperature sensitive resistors control the rate of heat flow). The

exact rates that the heat is supplied to the sample and reference areas, to achieve a given heating rate, depend on the heat capacities of the sample and reference.

Proponents of DSC or DTA analyses argue as to the relative advantages of one over the other, although with updated instrumentation containing microprocessors, computerized transformations can be made to give similar data. DTAs ideally cover wider temperature ranges, while DSCs are better at very low heating rates. In favor of DSC, one instrument constant can be used across all temperatures since sample and reference are maintained at the same temperature. To calculate enthalpy changes from DTA data, a calibration constant is needed, which is a function of temperature and is influenced by the heat capacities of the sample and reference materials. The temperature of transition can be accurately determined by DTA. For DSC, the temperature and enthalpy (ΔH) of transition can be directly measured since two heaters, for sample and reference, maintain equal temperature. The rate of differential heat input (dH/dt) plotted versus temperature or time can be monitored for the whole transition. The remainder of this chapter will primarily describe the DSC instrumentation and applications.

36.2.3 Differential Scanning Calorimeters

For proper use of a DSC, it is important that the experimental conditions be standardized for each series of experiments. The following are some of the conditions that influence instrument output:

1. Sample pan size, material, and its resistance to corrosion
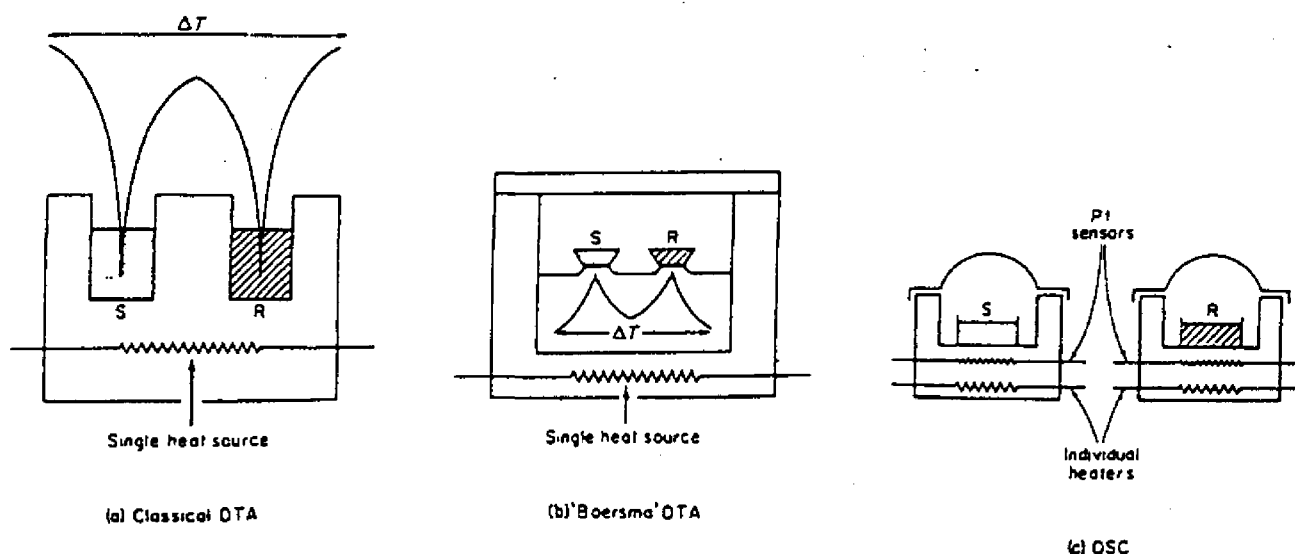
36-1 figure Schematics of typical configurations in differential thermal analyzers: (a) classical differential thermal analyzer (DTA), (b) Boersma DTA, (c) differential scanning calorimeter analyzer (DSC). [From (2), used with permission. Courtesy of Perkin-Elmer Corporation, Norwalk, CT.]

2. Heating rate of scan
3. Placement of sample pan inside sample holder
4. Furnace atmosphere
5. Size and shape of the furnace

The following sample properties can affect instrument output:

1. Weight (sample size)
2. Layer thickness in pan
3. Particle size
4. Packing
5. Thermal conductivity
6. Heat capacity
7. Placement of sample inside sample pan

To gather, interpret, and calculate the proper onset, peak, and end temperatures and heat of transition, as well as the heat capacity of the sample, the instrument must be calibrated with the aforementioned well-characterized standards, such as indium. Indium has a ΔH of fusion of 28.4 J/g, m.p.156.64°C (6,7). This information can be used to calculate the calibration constant of the instrument from the following equation:

$$K_R = \Delta H_{indium} \times W_{indium}/A_{indium} \qquad [4]$$

where:

K_R = calibration constant at a given scan rate, R

ΔH_{indium} = enthalphy per gram of the heat of fusion for indium

W_{indium} = weight of indium in the sample pan

A = area of indium heat of fusion peak for the weight of indium used on the DSC used

Then the enthalpy of sample is:

$$\Delta H_{sample} = (K_R)(A_{sample})/W_{sample} \qquad [5]$$

where:

A_{sample} = area of the transition peak for the sample at scan rate R

W_{sample} = weight of the sample

In this way, the area under the peak for an unknown transition can be used to calculate the ΔH of the transition, since in DSC work the area does not include the heat capacity.

The usual sample size (6–12 mg) can be placed in either small (up to 20 mg) volatile or nonvolatile sample pans (usually sealed volatile sample pans are used in food science work) or stainless steel capsules that can withstand high-pressure buildup inside them, such as that caused by the volatilization of water. The

high-pressure capsule can accommodate larger sample sizes (up to 45 mg). Volatile sample pans (often called hermetic pans) are used most commonly with food samples and are made of aluminum. They come with lids that are crimped (cold sealed) with a special tool to ensure a good seal. The internal pressure that volatile sample holders can withstand is only two to three atmospheres. The high-pressure capsules often are stainless steel and can withstand up to 30 atmospheres pressure. Care should be taken in the use of high-pressure capsules because they can injure the operator or instrument sample holder assembly (thermal head) if they explode.

The heating rate should be slow enough to obtain distinct and reproducible peaks for each transition. Commonly, scans are from 1 to 10 degrees/min. The apparent temperature of the transition is influenced by the sample size due to thermal lag. If the sample is much greater than 20 mg, or if the sample pan does not have good thermal contact with the holder that is touching the heater, uniform thermal conductivity may not take place. This will result in nonreproducible data. In addition, if the entire assembly is dirty, the data may be indicative not of the sample but of the contaminants.

Since DSCs measure heat flow, larger samples and faster heating rates will give larger signals. However, too large samples and too fast heating rates also broaden transitions. In general, it is best to use the minimum sample size and slowest heating rate that is practicable to give the desired resolution of transitions and thermal data.

In actual practice, DSC thermograms can become quite complex, with many thermal events occurring at the same time. A recent addition to DSC instrumentation available to the researcher that addresses this complexity is the modulated differential scanning calorimeter™ (MDSC™) (8, 9). The MDSC cycles, or modulates, the temperature in a sinusoidal manner while the net temperature increases at a specified rate. The result is that reversible and irreversible thermal changes can be separated. For example, glass transitions, which are reversible, can be separated from other irreversible effects, such as gelatinization, recrystallization, and protein denaturation. An example of the use of MDSC is shown later (Fig. 36-14).

Once the scan is completed, the remaining problem is the proper interpretation of the data obtained.

36.2.4 Data Interpretation

The following examples of DSC heating curves, adopted from the Perkin-Elmer instruction manual for the DSC-2 (10), show differences due to changes in heat capacity (C_p) and the peak thermal transitions. Figure 36-2A shows no change in heat capacity across the transition; Fig. 36-2B shows a broad transition (where the baseline may not be flat); Fig. 36-2C shows a transi-

that might result from a DSC heating scan. ICTA defines the area under the curve as ABCA. An open question is whether the onset temperature is at A or D. One author of this chapter (TWS) prefers to report the extrapolated onset temperature (D) instead of the first deviation from the baseline (A). The precise value would depend on whether there is a sharp versus a broad transition, or whether there is a single transition. The peak temperature for melts has been interpreted as the point when all the material has melted. However, it has been shown from other types of measurements that the peak temperature does not indicate either the maximum rate or the end of the transition being monitored.

Most researchers agree onset temperature is more significant than peak temperature, since peak temperature is greatly influenced by scan rate and sample size and does not always relate to a specific physical change. Although one may report peak temperatures to compare to other reports, onset temperatures should be used to interpret data.

36-2 figure Stylized curves one might find in differential scanning calorimetry (DSC) scans. (A) Curve with no change in heat capacity, (B) a broad transition (baseline may not be flat), (C) a transition with a concomitant change in heat capacity, (D) heat capacity change during a glass transition, (E) the effect of an increase in sample size (dashed line) on the transition, (F) comparison of a primary thermogram and its first derivative (dashed line). [Adapted from (10). Courtesy of Perkin-Elmer Corporation, Norwalk, CT.]

tion with a change in heat capacity; Fig. 36-2D shows a change in heat capacity during a glass transition, and defines its transition temperature (some researchers report the onset temperature as for other transitions); Fig. 36-2E shows how an increase in sample size affects the transition peak temperature (hence it is better to report onset temperature); Fig. 36-2F compares a primary thermogram with its first derivative (dashed curve). Some researchers use the first derivative curve to better interpret onset, peak, and end temperatures.

Figure 36-3 is a composite stylized set of thermal output. The directions of the endothermic and exothermic heat flows are labeled on the y-axis, and the temperature increases from left to right. This output can be interpreted as a rise due to the heat capacity (C_p) of the sample as the scan starts from the isothermal state, followed by a ΔC_p due to a glass transition, an exothermic crystallization, then a broad endothermic peak due to temperature-dependent phase changes, and ending with a return to isothermal conditions.

One major controversy in the interpretation of DSC curves relates to the onset temperature, the meaning of the peak temperature, and the determination of the baseline. Figure 36-4 shows a stylized thermal peak

36.3 APPLICATIONS

DSC is widely applied in the food industry to interpret water, starch, protein, lipid, and carbohydrate interactions. There will be numerous studies in this area, and a few examples are given below.

The amount of freezable versus nonfreezable water in the system can be evaluated. Figure 36-5 shows an example of the ice-melting endotherm and how it is affected by the presence of starch, after gelatinization has taken place (11). The larger the ice-melting endotherm, the greater the amount of freezable water in the system. As would be expected, the endotherm for pure ice is larger than that of ice in the presence of starch after gelatinization. Also, there might be a shift in the onset temperature due to the presence of increasing amounts of water-soluble solutes.

Starches can be studied in many different ways by DSC. The influences of the amount of water, type of starch, and other components present with the starch have been studied extensively for the last 20 years. Examples of the enthalpy of potato starch gelatinization as a function of percent moisture can be found in Fig. 36-6 (12). One can see from Fig. 36-6 that as the volume fraction of moisture goes below 0.81, the original peak decreases and a shoulder develops. The shoulder is shifted to higher temperatures as it becomes the main peak. Finally, the enthalpy of the higher temperature endotherm decreases as the water content of the sample is further reduced.

An example of using DSC to study glass transitions of wheat starch can be seen in Fig. 36-7, from the work of Zeleznak and Hoseney (13). In thermograms of native wheat starch at various water contents below 30%, one can see onset temperature (T_o) shifting to
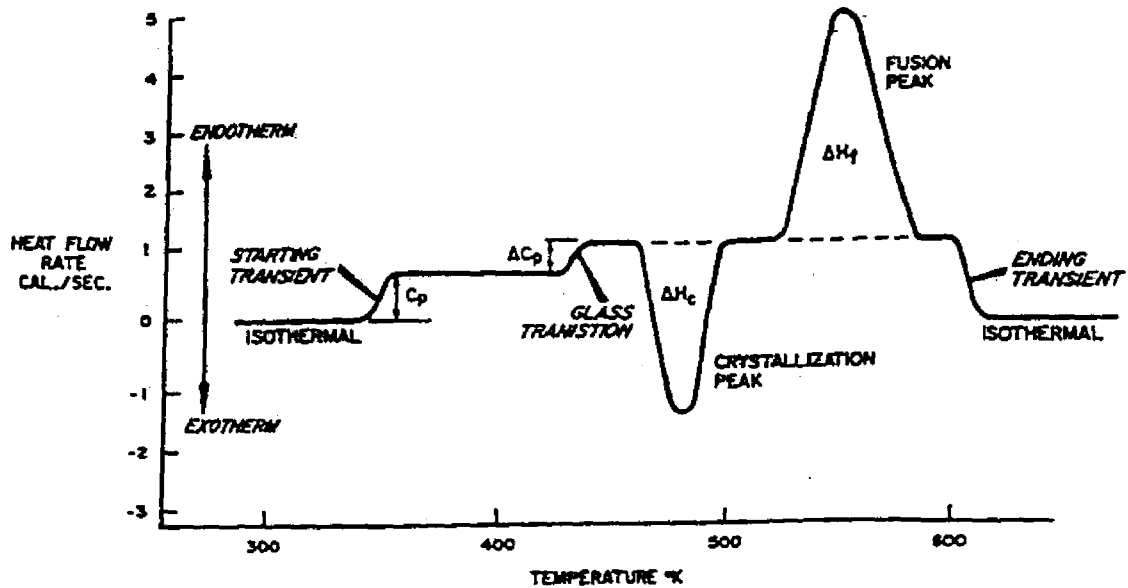
A set of thermal transitions as they might appear when a sample is heated. [From (10), used with permission. Courtesy of Perkin-Elmer Corporation, Norwalk, CT.]
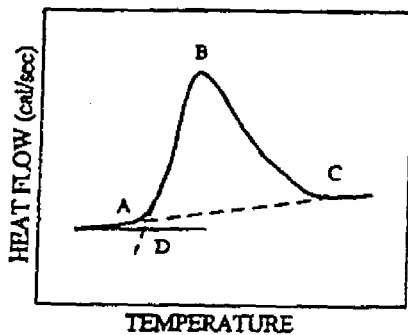
A stylized thermal peak indicating possible ways to report thermal transition temperature (A → D).

higher temperatures with decreasing water content, and glass transitions (T_g) can be seen readily above 20°C, below 20.1% water content.

Chemical modification affects the gelatinization of native starches, as can be seen from Fig. 36-8, in which waxy and normal corn starches were phosphorylated, octenylsuccinylated, hydroxypropylated, acetylated, or quaternary ammoniated (14). In addition, corn starch gelatinization can be delayed by addition of different concentrations of sucrose, as seen from the work of Johnson et al. (15) in Fig. 36-9.

Polymorphism of mono- and triglycerides has been recognized for many years. Such molecules play an important role in the functionality of emulsifiers in foods. Generally, triglycerides have been shown to exist in an α, β, and β' forms. Within those categories,
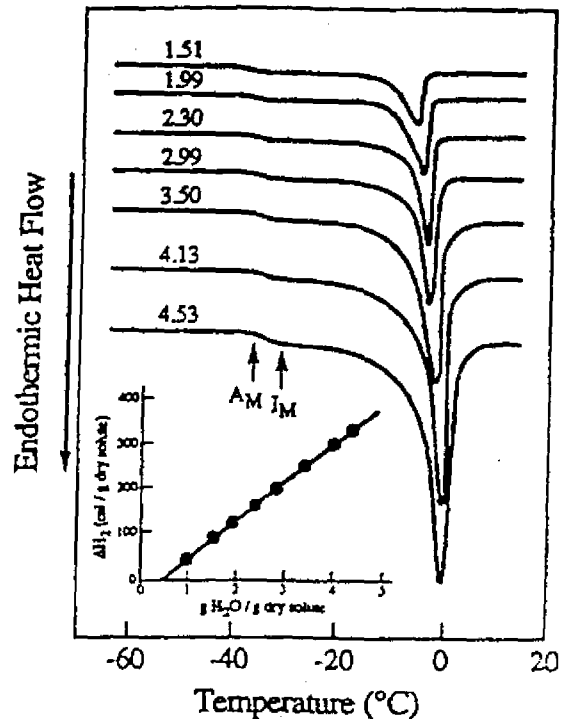
Thermograms of ice melting in the presence of different amounts starch. [From (11), used with permission.]

one can find polymorphic forms. The physical and thermodynamic behavior identifies the various polymorphic forms. For example, Simpson and Hagemann (16) have shown that tristearin can exist in an α, β, and
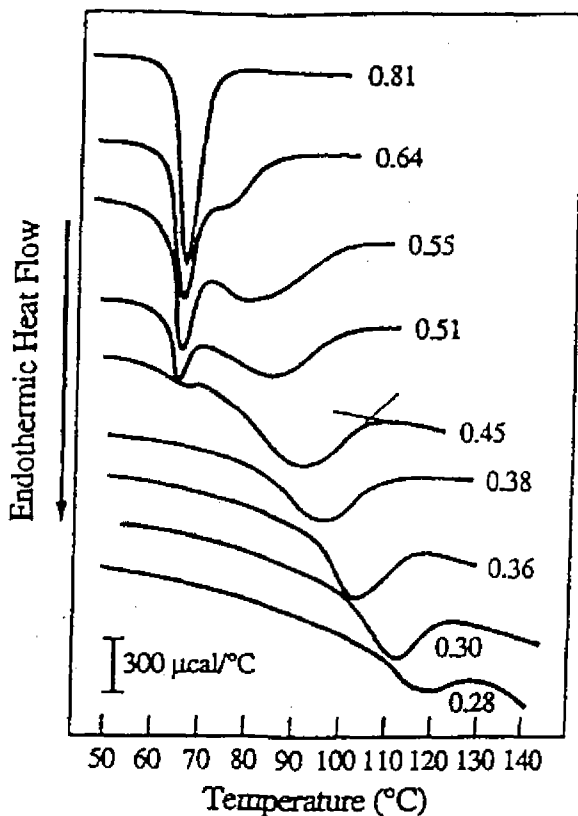
β'_1, β'_2. From Fig. 36-10, one can see that tristearin, when heated, gives different DSC curves. An exothermic reaction takes place during its conversion from the α to the β phase at 55°C. However, β'_1 and β'_2 convert to the β phase at 64°C differently. The β'_2 shows a small endothermic peak at 61°C prior to the phase change at 64°C, while the β'_1 does not. If the triglyceride is all in the β phase prior to heating, no conversion is seen by DSC. Therefore, Simpson and Hagemann could not show a DSC scan for the β form during heating.

Emulsifier phase changes can be studied by DSC either alone or in the presence of other components (17). In Fig. 36-11, one can see saturated monoglyceride transitions without water or sucrose (Fig. 36-11a); in the presence of 42% sucrose solution (Fig. 36-11b); and in the presence of pure water (Fig. 3-11c). Figure 36-11a', b', and c' are rescans of Fig. 36-11a, b, and c after cooling.

Meat scientists use DSC to study the thermal denaturation of muscle. An example of such an application can be seen in Fig. 36-12 in which post-rigor beef and cod muscle differ in their thermograms (5). The
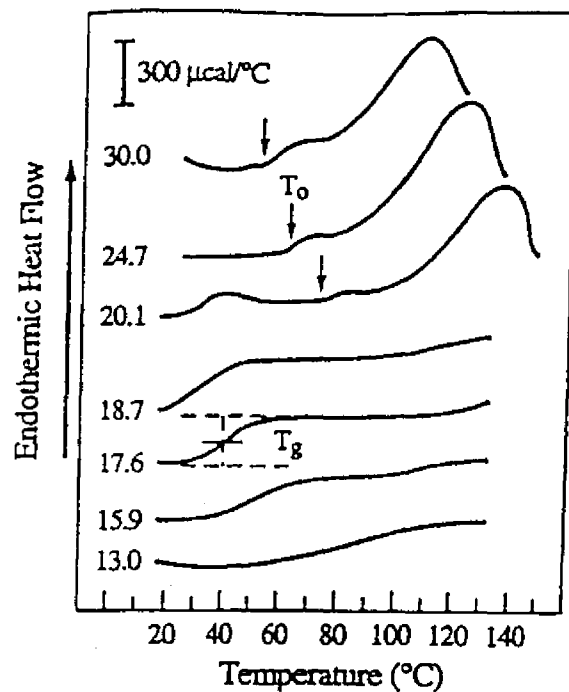
three peaks refer to denaturation of the head, tail, and a flexible region of myosin. These denaturation are strongly influenced by ionic strength and other factors.

DSC can be used also to compose the phase diagram for a solute–solvent system. This is done by measuring the types and temperatures of transitions of various compositions of a system. For most food systems, however, even simple two-component systems, such as sucrose–water, give transitions that are dependent on heating/cooling rate. Thus the diagram that is composed is often called a "state" diagram (18), indicating that we are observing only one state of the system for these experimental conditions. Nonetheless, such state diagrams are useful for guiding the food scientist in areas such as spray-drying, freeze-drying, caking, softening, extrusion, etc. An example of using such data is shown in Fig. 36-13, in which the DSC (and glass transitions) of grapes with various water concentrations are shown. Figure 36-14 shows the resulting state diagram of the grape system.

The use of modulated DSC (MDSC) is shown in Fig. 36-15 for a low moisture polydextrose sample (19). A reversible glass transition is clearly separated from other thermal events, making this a useful technique to understand complex thermograms.

Further applications of DSC to food research can be found in numerous articles. Biliaderis has published a review that gives numerous examples of interest to food scientists (20).

36-15 figure MDSC thermogram of polydextrose containing 4.0% moisture. (a) Total heat flow; (b) irreversible heat flow; (c) reversible heat flow. [From (19), used with permission.]

related research are given in this chapter. The application examples include water, starch, protein, lipid, and carbohydrate transformations and interactions.

36.5 STUDY QUESTIONS

1. What types of information can you obtain from thermal analysis work? What types of thermal analyzers might you use for each of the thermal analyses listed in the text?
2. Define differential scanning calorimetry and how it differs from differential thermal analysis.
3. The glass transition temperature (transformation from the glassy to rubbery state) of a food material is needed for an extruded product so that the operator of the extruder can set the operating temperature most efficiently. Sketch what the DSC curve of this material might look like.
4. What sample constraints are needed to optimize DSC or DTA signals?
5. How could you quantify the amount of energy required for a molecule to undergo a thermal transition?
6. Starches are often characterized by observing the viscosity behavior of a starch slurry as the temperature is increased. Explain how this is a form of thermal analysis.
7. You have a mixture of a fat (10%), protein (5%), and water (85%). Sketch what type of DSC curve you might obtain if you scanned from -30°C to 120°C, and explain why that curve might be obtained.

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37 chapter

Color Analysis

F. J. Francis

37.1 INTRODUCTION

The three major aspects of food acceptance are color, flavor, and texture. Many colorimetrists believe that color is the most important because if a product does not look right, a consumer may never get to judge the other two aspects. Regardless, color is one of many aspects of appearance such as gloss, particle size, physical state, background, illumination, etc., but it may well be the most important.

Color judgments data back to antiquity because of the obvious impact of colored scenes and objects in history. The psychological importance of color led to the development of many visual systems of characterizing color. With the development of the sciences of physics and electronics, it became possible to develop instrumentation to duplicate the color responses of the human eye. Research in vision physiology demonstrated that the human eye could theoretically differentiate between 10,000,000 colors, and instrument makers considered this to be unfair competition. The development of electronics has made it possible to develop a wide array of colors. For example, fashion designers have sophisticated methods for reconstructing and retouching photographs. For the color aspects alone they can generate a theoretical 16,000,000 colors. But, obviously, many of these are below the threshold of visual discrimination of the human eye.

Color is not a physical attribute such as melting point or particle size. Rather, it is one portion of the input signals to the brain that reacts to produce the perception of appearance. Color, as seen by the eye, is an interpretation by the brain of the character of light coming from an object. It is possible to define color in a purely physical sense in terms of the physical attributes of the food, but this approach has serious limitations when we try to use color measurement as a research or quality control tool in food processing or merchandising. A more satisfactory approach is to define color in a physical sense as objectively as possible and interpret the output in terms of how the eye sees color.

The measurement of color in foods today is a mature science and we can easily measure the color of almost anything. This chapter is devoted to the acquisition, understanding, and use of relevant color data for food products.

37.2 PHYSIOLOGICAL BASIS OF COLOR

It is possible to estimate rigorously the physical stimuli received by the human eye, but unfortunately this is not true for physiological reactions. The initial stimuli by which we perceive color have been well described and documented in the early literature (1–3). In brief,

the eye has two types of sensitive cells in the retina, the rods and the cones (4). The rods are sensitive to lightness and darkness and the cones to color. There are three types of cones within the retina, one sensitive to red, another to green, and the third to blue. It has been known for over a hundred years that there had to be three types but only recently has it been possible to demonstrate it anatomically. Even more recently, at least nine genes were demonstrated to control the formation of the cones and two produce slightly different red-sensitive cones; thus it is likely that individuals differ in the way they see color. But the differences must be small because in 1931 an international group called the Commission Internationale d'Eclairage (CIE) was able to define a "standard observer." Essentially it was the average response of 92% of the population with normal color vision. The variations in individual responses were remarkably small in view of the variation in individual responses in taste and smell. The cones send a signal to the brain that sets up a response in terms of opposing pairs. One pair is red-green and the other is blue-yellow, and this is why we have individuals who are red-green or blue-yellow color blind. There are no individuals who are blue-green or red-yellow color blind.

The interpretation of signals to the brain is a very complex phenomenon and is influenced by a variety of psychological aspects. One such aspect is color constancy, since a sheet of white paper looks white in bright sunlight and also when it is under the green leaves of a tree. The physical stimuli in each case are obviously quite different, but the brain knows that the paper should be white and draws on its experience. A second aspect occurs when a large expanse of color appears brighter than the same color in a small area. One needs only to paint a whole wall of a room and see how different it appears from a small color chip in the paint store. There are many examples of this type of interpretation of color by the human brain. The old adage "I believe what I see" is interesting but unfortunately not always true since it is a simple matter to fool the human eye. A classic example shows a triangle with three right angles. This is obviously impossible and it is only when we see a view from another angle do we realize that the sides of the triangle do not meet in space. In this situation the brain was not given sufficient information to make a correct judgement. But the brain will make a judgment, based on available information, which may, or may not, be correct.

37.3 DEVELOPMENT OF COLOR SYSTEMS

Color systems are ways to describe color. Such systems include verbal or numerical designaations for visual

matching of colors, and mathematical terms used with instrumentation.

37.3.1 Visual Systems

Historically color has been estimated visually and a number of well designed and well researched color atlases have been produced (5, 6). The most well known are the Munsell System, Hungarian Coloroid System, the German DIN System, the Inter Society Color Council/National Bureau of Standards System, the Argentinian Villalobos Color Atlas, the Natural Color System, and the Ostvald System. In terms of food applications in the United States, the Munsell System is probably the best known. Munsell was a Boston artist who wanted to teach his students about color rendition. He realized, as did others before him, that color was three-dimensional. This aspect may be visualized by referring to a citrus orange fruit. The core up the center may be likened to Munsell Value V (representing lightness or darkness). Each section around the circumference may be Munsell H (for hue) such as red, green, blue, etc. The distance from the core to the edge may be Munsell C (for chroma) and refers to the intensity of color. The HVC parameters are shown in a vertical slice through the solid (Fig. 37-1) and a horizontal slice (Fig. 37-2).

The Munsell color atlas contains 1225 colored chips each with a numerical designation. The chips are
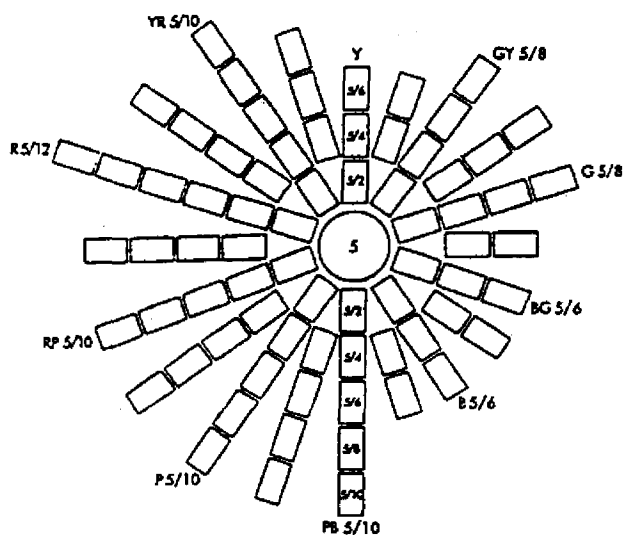
37-2 A horizontal section through the Munsell color system solid at Value 5.

37-1 A vertical section through the yellow–purple blue plane of the Munsell color system solid.

normally 5/8 by 7/8 inches in size but samples of any size up to 8 1/2 by 11 1/2 are available (7). A number of specialized arrays also are available for color specification of materials such as hair, skin, soil, grass seed, rocks, textiles, insulated wire, and cable (3), and A.S.A. (American Standards Association) safety colors, etc. When a color is specified by its Munsell designation, the color is unambiguous.

The designation of the color of a food by visual comparison with chips from a visual color solid is very appealing, since it is simple, convenient, and easy to understand. Many specialized color standards of paint, plastic, or glass are available for use as food standards (3, 7), and a number of companies have adopted this approach for food quality purposes. For example, the official USDA grading system for tomato juice employs spinning discs of a specific Munsell designation to describe the color grades (7). Glass color standards are available for sugar products. Plastic color standards are available for a large number of commodities such as lima beans, peas, apple butter, peanut butter, orange juice, canned mushrooms, peaches, sauerkraut, salmon, pimento, etc.

The glass and plastic color standards have been very successful, but obviously are available in a limited number of colors. Painted paper chips, such as the Munsell system, are available in a much wider range of colors, but even these are limited. They are fragile and may change with use. The visual color standards also have another problem in that they are tiring and sometimes tedious. Colors that fall between existing standards are sometimes difficult to convey to other indi-

viduals. These are the main reasons why instrumental methods have been so appealing.

37.3.2 Spectrophotometric Approaches

37.3.2.1 Historical Development

The early instrumental methods for color measurement are based on transmission or reflection spectrophotometry (6, 8). The concepts were based on research by the physiologists who developed the responses of the cones in the human eye in terms of the visible spectrum. They were able to do this in a manner easily reproducible in the laboratory today. Three projectors are required, with a red, green, or blue filter in front of the lens (Fig. 37-3). The resultant red, green, or blue light beams are focused on a screen such that they overlap over half a circle. The other half is illuminated by another projector or by spectrally pure light from a prism or grating such that the observer can see both halves of the circle on the screen simultaneously. Each projector is equipped with a rheostat to vary the amount of light from each of the red, green, and blue sources. By varying the amount of light, the observer can determine the amounts of red, blue, and green required to match almost any spectral color. Therefore we can define spectral color in terms of the amounts of red, green, and blue (RGB) (7). We can set up an equilateral triangle with one of the RGB stimuli at each corner, as shown in the diagram on the right in Fig. 37-4. Figure 37-4 also shows the RGB diagram as a right-angled triangle in the diagram on the left. Figure 37-5 shows the RGB diagram in more detail. In this case, the amount of blue is obtained by subtracting the amount of red and green from unity. Every point within the triangle represents a color and can be specified mathematically by the amount of red, green, and blue. Unfortunately, red, green, and blue are not particularly good stimuli to use, since not all colors can be matched with them. The early researchers were asked to choose a set of coordinates that would be more appropriate and they chose XYZ. They cannot be reproduced in the laboratory since they are only mathematical concepts. If one wants a crude visual reference, one can think of X as red, Y as green, and Z as blue. The relative positions in space for red, green, and blue and X, Y, Z are shown in Fig. 37-4. The X, Y, and Z coordinates were selected for a number of mathematical advantages that are beyond the scope of this chapter (9).

37.3.2.2 The Standard Observer

If we take the red, green, and blue data for the spectral colors, transform them to X, Y, and Z coordinates, and plot the responses of the human cones against wave-
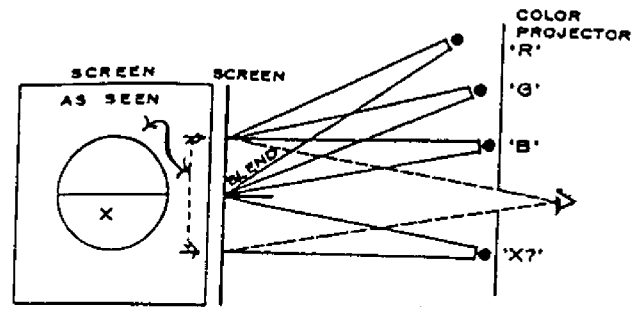
37-3 figure Diagram showing three projectors focused on the upper half of a circle on the screen. The color to be measured is projected on the lower half and the eye can see both halves simultaneously.
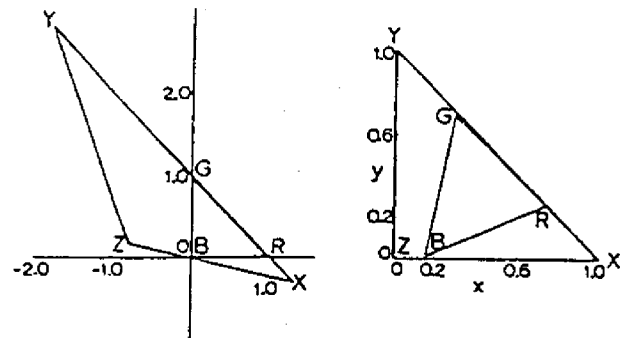
37-4 figure Diagram showing the relative positions in space of the RGB and XYZ stimuli. The diagram on the left shows the GRB coordinates as a right-angled triangle. On the right, the XYZ coordinates are plotted as a right-angled triangle.

R=700
G=546
B=436
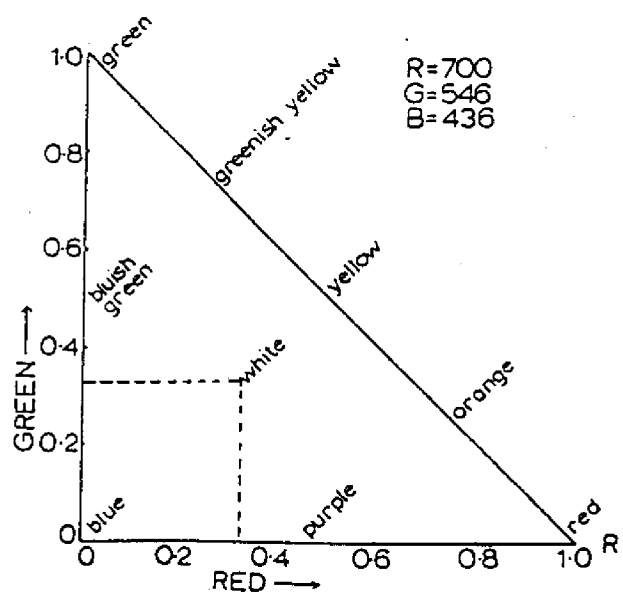
37-5 figure Colors plotted on a red, green, blue color triangle.
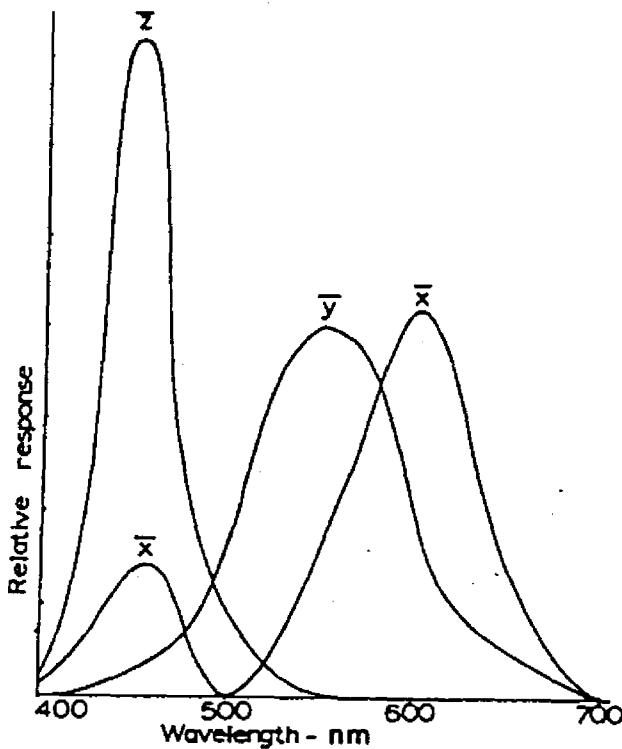
The standard observer curves relationship between the response of the human eye, defined as the standard observer curves ($\bar{x}$, $\bar{y}$, $\bar{z}$), and the visible spectrum.

length (Fig. 37-6), we have the responses of the human eye to color. These curves were standardized in 1932 and were called the CIE $\bar{x}$, $\bar{y}$, $\bar{z}$ standard observer curves. They made it possible to calculate color coordinates from spectrophotometric data.

37.3.2.3 The CIE System

Color data can be calculated from a reflection curve, or a transmission curve, from a sample by integrating the area under the curve in terms of the light source and the standard observer curves.

$$X = \int_{380}^{750} R\, E\, \bar{x}\, dx \qquad [1]$$

$$Y = \int_{380}^{750} R\, E\, \bar{y}\, dy \qquad [2]$$

$$Z = \int_{380}^{750} R\, E\, \bar{z}\, dz \qquad [3]$$

where:

R = the sample spectrum
E = the source light spectrum
$\bar{x}$, $\bar{y}$, $\bar{z}$ = the standard observer curves.

The XYZ data usually are plotted as x, y, z coordinates, where:
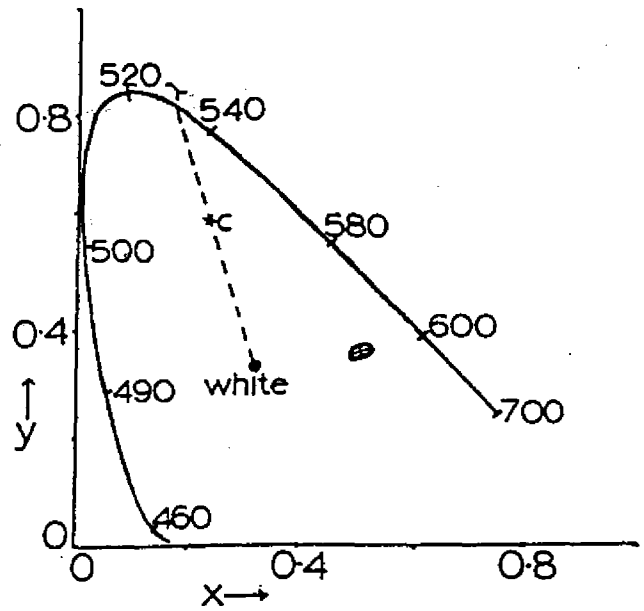
The spectrum colors plotted on x, y coordinates.

$$x = \frac{X}{X + Y + Z} \qquad y = \frac{Y}{X + Y + Z}$$

$$z = \frac{Z}{X + Y + Z} \qquad [4]$$

The spectrum plotted on an x, y diagram is shown in Fig. 37-7. The color solid is actually a solid, not a plane, with the lightness function perpendicular to the plane of the paper. Figure 37-7 also illustrates another popular way of presenting color data. The point of intersection of a line from the coordinates of white light (x = 0.333, y = 0.333) through the point to the edge of the solid is the dominent wavelength of the color. The relative distance from white light is the purity of the color.

The early spectrophotometers provided a reflection or transmission spectrum and the XYZ data had to be calculated by hand. This was very tedious so mechanical integrators were developed that were later replaced by electronic integrators. However, these instruments were usually complicated and expensive and this facilitated the development of tristimulus colorimeters.

37.3.3 Tristimulus Colorimetry

The definition of the standard observer led to the development of colorimeters designed to duplicate the response of the human eye. The concept is very simple (Fig. 37-8). One needs a light source; three glass filters with transmittance spectra that duplicate the X, Y, and Z curves; and a photocell. With this arrangement, one
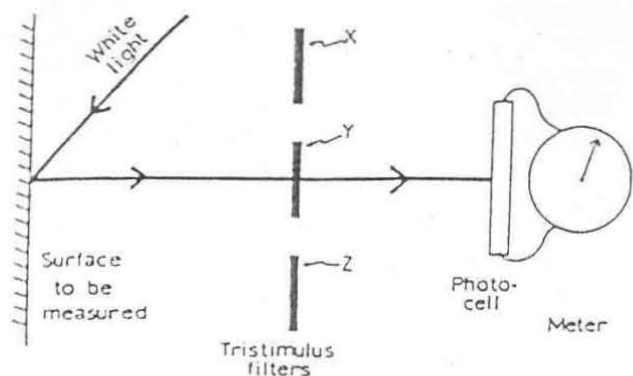
37-8 figure Essential components of a tristimulus colorimeter.
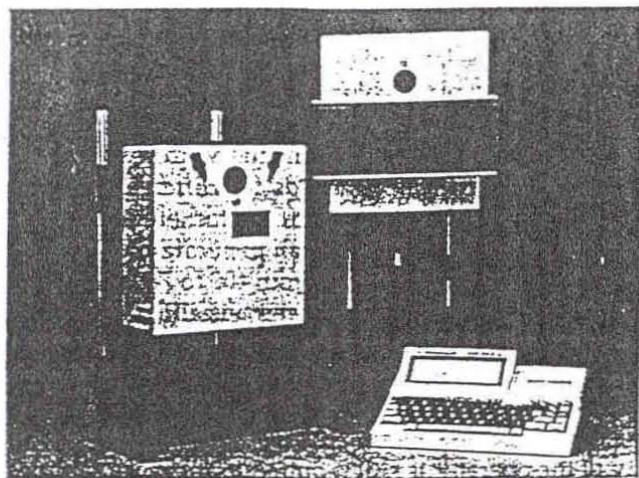
37-9 figure A tristimulus colorimeter with normal- and large-area viewing sensors. (Courtesy of Hunterlab, Reston, VA.)
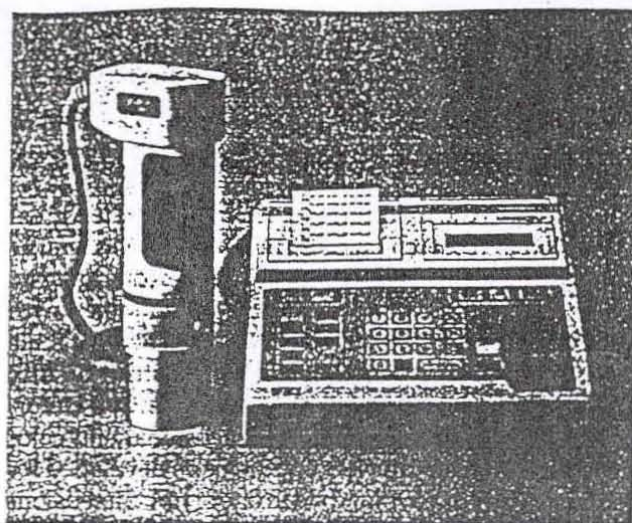
37-10 figure A tristimulus colorimeter—the Minolta Chroma Meter. (Courtesy of the Minolta Co., Ramsey, NJ.)
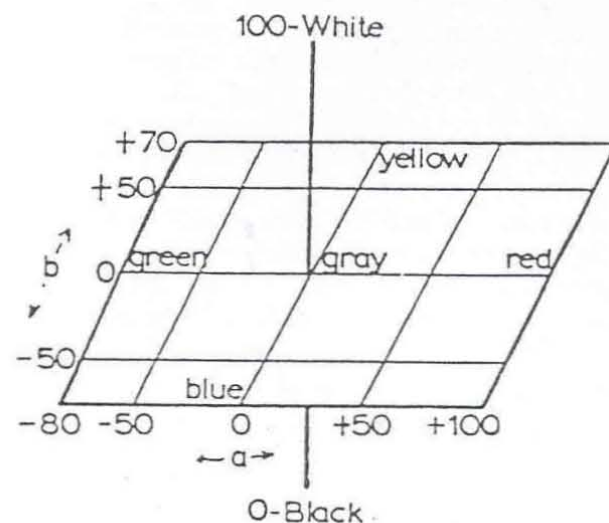
37-11 figure The Judd–Hunter color solid.

can get an XYZ reading that represents the color of the sample. All tristimulus colorimeters today depend on this principle with individual refinements in photocell response, stability, sensitivity, and reproducibility (Figs. 37-9 and 37-10). However, in spite of the similarity in concept, they do not all use the same units. It is possible to use a number of different filter–photocell combinations as well as different axes in color space and even different color solids. [A color solid refers to a three-dimensional space bounded by physical (color chips) limits or mathematical concepts. Color space refers to the space within a color solid.] However, some standardization is developing and four instrumental color systems seem to be gaining in popularity.

One of the instrumental color systems is the CIE-XYZ system and a second is the Judd-Hunter $L\,a\,b$ solid (Fig. 37-11). The latter represents a color solid, where L = lightness or darkness, $+a$ = redness, $-a$ = greenness, $+b$ = yellowness and $-b$ = blueness. A third

scale is known as the CIELAB system with parameters $L^*a^*b^*$. A fourth scale is the CIELCH system with parameters $L^*C^*H^*$. The second and third systems were developed primarily to produce a system that was visually more uniform throughout the whole color solid. The fourth is a polar system with primary emphasis on color tolerances. (Another system called CIELUV was designed primarily for use with lighting and television and has not found much application with foods.)

Regardless of the particular instrument, or the mathematics of the color system involved, the limiting factor is the ingenuity of the operator in getting a representative signal from the food in question and inter-

preting it in terms of color. The data are empirical, but nonetheless useful for characterization, process control, quality control, purchase specifications, etc.

37.3.4 Specialized Colorimeters

The success of the tristimulus colorimeters led to a great expansion in research on color measurement as well as the manufacture of a number of different colorimeters. Color data were now easily and quickly obtained with relatively inexpensive instrumentation. The time scale coincided with the development of statistical quality control (SQC) concepts for production control. Unfortunately, SQC charts were two-dimensional and color data came in three dimensions. Requests developed for rationales to reduce color data to one or two dimensions and a series of specialized instruments were developed. One of the earliest was the tomato colorimeter (10) designed to measure the color of raw tomato juice. The impetus for the development of this instrument was for incentive payments for growers to deliver more highly colored tomatoes to the processors.

The development of the tomato colorimeter provides an interesting model. Samples of tomatoes representing the range of commercial samples were graded by USDA inspectors into grades A, B, and culls. The juice then was extracted from the tomatoes and measured on a tristimulus colorimeter. A relationship then was established between the grader's decision for the raw tomatoes and the color of the juice. In effect, the equation representing how the graders visualized the color of the tomatoes was established in color space. In this application, tomato color (TC) was represented by:

$$TC = 2000 \cos \theta / L \qquad [5]$$

where:

$$\cos \theta = \frac{a}{(a^2 + b^2)^{1/2}}$$

The Lab units are in the Judd–Hunter system

This instrument became known as the USDA Tomato Colorimeter. It proved to be a useful instrument and it was soon modified to read the color of processed tomato juice according to the equation:

$$\text{Color Score} = bL/a \qquad [6]$$

The same approach was used to develop the Citrus Colorimeter to measure the color of orange juice. Specialized instruments were developed for honey, sugar, tea, apples, cranberries salmon, wine, internal color of pork and beef, etc. (11). It may be stretching a point to say that all the instruments measured color as such, since they were all concerned with the general aspect of

quality, but color was a major factor. The proliferation of specialized instruments led to some dissatisfaction since, for example, suppliers did not want a roomful of specialized equipment. Another approach gained in popularity. When the original data from a sample are collected in spectrophotometric or tristimulus units, it can be read out in any desired units by a simple microprocessor in the unit or the software of a computer. For example, the scales for raw and processed tomato juice can be read from the same instrument with an extra circuit. This trend has discouraged the accumulation of data in other than fundamental units such as the four listed previously.

The design of instruments to measure color has come full circle. The first instruments were spectrophotometers but the labor in calculation was so high that the tristimulus colorimeters were developed. Then the development of electronic calculation became so efficient that the labor of calculation ceased to be a factor. Today most of the color measuring instruments are spectrophotometers. Current instrumentation ranges from relatively simple instruments with a variety of exposure and measuring heads for different applications to sophisticated spectrophotometers coupled with a computer. The latter can generate data in four viewing systems, reflectance from 400 to 700 nm, seven color scales, 15 specialized scales, six illuminants,and actually any conceivable memory and readout desired. All instruments use computerized feedback to minimize drift and light source fluctuations. The measurement of color may be a mature science but the ingenuity of the operator is still required to measure samples such that meaningful data can be obtained.

37.4 MANIPULATION OF DATA

36.4.1 Presentation of Samples

Nearly all of the general purpose instruments today are designed to maximize the collection of light from the sample. The relationships between the light signal and the interpretations of color by the brain have been standardized for ideal situations for both major modes of measurement, namely perfect transmittancy and complete reflectance. Unfortunately most foods lie between the two ideals and both transmit and absorb light. This lends some empiricism to the data. Figure 37-12 shows a typical presentation for a food sample for measurement by reflection. Light enters from the source beam below and some light is reflected from the glass surface into the measuring port of the instrument. Some of the light enters the sample and is scattered. Part of the scattered light is reflected back into the measuring port, but some escapes through the sides of the cell. Light entering the sample is usually both partially scattered and absorbed, and the instrument measures
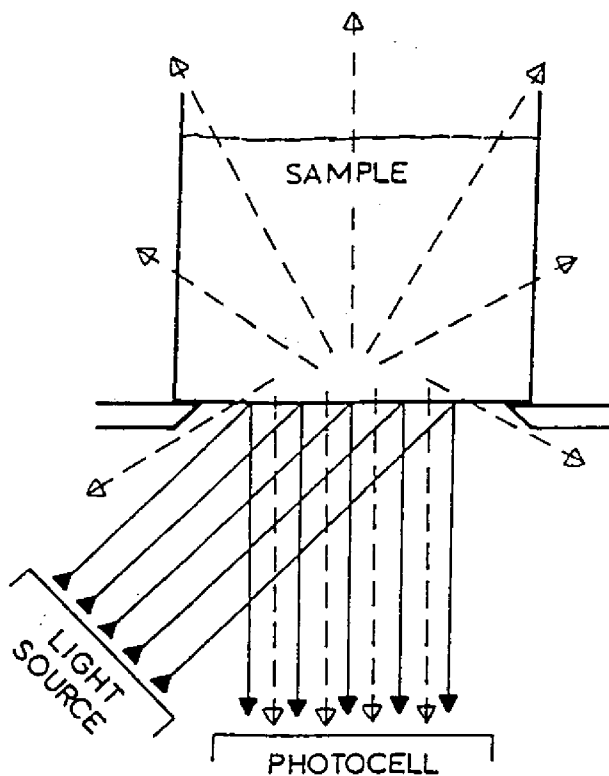
Diagram showing the interaction of a light source with a sample to produce a signal.

the light that emerges back into the measuring port. It is obvious that the instrument response is empirical but it is usually reproducible for a given situation.

Turbid solutions may show less reproducibility in color measurements since the turbidity may not be uniform or reproducible. The problems with light scattering and absorption can be handled by the Kubelka–Munk equations (9, 12) but they have not been accepted to any extent by the food industry. The particles could be filtered out prior to measurement but this would change the visual appearance of the product. The simplest approach to obtain a reproducible reading is to measure the color by reflectance from the bottom of a cell filled to infinite thickness such that any increase in depth of sample does not change the reading. This does not correct for the problems in light scattering and absorption but it usually assures a reproducible and empirical reading. With granular samples, it is important that the size of the particles be standardized since usually small particles appear whiter than larger particles. With samples that show directional differences, such as strands of spaghetti, some judgment is required to determine whether the sample should be measured parallel to the strands, or at right angles,or both. With this situation, instruments with large area illumination in a circular nondirectional

mode would be the method of choice. Some samples may show textured differences when the position of the sample is rotated and this situation requires judgment about how the data will be used. One overall concept is to try to measure the color in a manner as close as possible to the way that the consumer sees the food.

37.4.2 Interpretation of Data

Interpretation of color with the visual systems is fairly straightforward. For example, the Munsell system has a gray sheet with a cutout the same size as a color chip. The sample can be placed beside the cutout in the Munsell book of color chips, under standardized lighting, and moved until a color match is found. The gray sheet removes the influence of the background.

Interpretation of data from instrument systems is more difficult. The early researchers set up the XYZ system for mathematical convenience and, given the XYZ cordinates of a color, it is difficult to visualize the color. Also, the discernible visual difference between two colors differed in size depending on the position in the color solid. These were the two major factors behind the development, and subsequent popularity, of the Lab system. A color with Lab cordinates is easy to visualize and more uniform throughout the color space. The CIELAB solid was even better. The early researchers programmed a computer to generate a color solid visually equidistant throughout the solid and it looked like a badly rumpled felt hat. Perfection may not be possible but the later color systems come close.

There is tendency of some researchers to publish color data as the actual three coordinates and do an analysis of variance on each of the three parameters. This approach is not recommended because an analysis of variance assumes that the components are independent variables. Actually they are not independent since both a and b depend on L. A better approach would be to calculate theta (θ). Theta is the angle that a line joining the point in Hunter space with the origin makes with the horizontal axis (Fig. 37-13). A θ value equal to zero would be a red color and θ equal to 90 and 180 would be yellow and green, respectively. Theta, as a measure of hue, can be interpreted visually and, together with L, can be used in an analysis of variance. Chroma also may be calculated by the equation:

$$\text{Chroma} = (a^2 + b^2)^{1/2} \qquad [7]$$

37.4.3 Reduction of Data

Reduction of data has received considerable attention in the quality control area since very few statistical quality control procedures are set up to handle three-dimensional data. The most accurate way to reduce the num-

$a/b = 0.2$

$a/b = 1.0$

C

D

E

THETA

yellowness
+ b

+ a —
redness
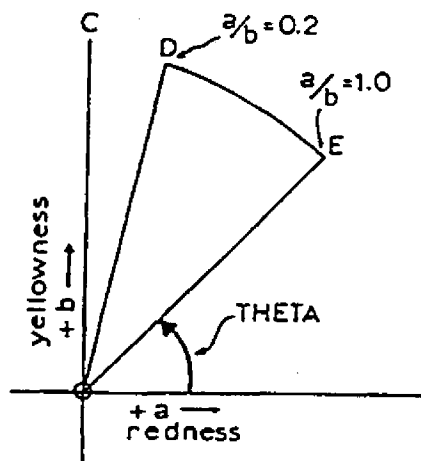
37-13
figure

Derivation of the hue function θ from a Hunter a, b diagram. Theta is a positive angle from 0 to 360 degrees.

ber of color parameters is probably via a regression equation. An example of this approach can be taken from the early work of Wenzel and Huggert (13) on the color of reconstituted orange juice. The correlation for reconstituted orange juice between USDA color score and Hunter readings for R_d (a scale related to L) alone was −0.815 and for a alone was 0.909. The correlation of the USDA color score with R_d and a was −0.927, and of the USDA color score with R_d, a, and b was 0.930. It was obvious that most of the correlation was with the a value and the inclusion of the other two in a multiple regression equation would result in some increase in accuracy, but the authors concluded that it was not worth the trouble. Similar work with lima beans indicated that the L value was the only important parameter whereas with tomato juice both a and b were needed. With applesauce all three parameters were needed to predict the subjective quality score. Reduction of data in terms of indices of fading, or "whiteness" (WI) is well known in the paper, paint, textile, and plastic industries and has had some applications to foods. Bolin and Huxsoll (14) used the following equation for lignin formation in abrasion-peeled carrots:

$$WI = 100 \left[\{100 - L^*\} + a^{*2} + b^{*2} \right]^{1/2} \qquad [8]$$

Reppond and Babbitt (15) used the following equation for whiteness of surimi:

$$WI = 100 - \left[\{100 - L^*\}^2 + a^{*2} + b^{*2} \right]^{1/2} \qquad [9]$$

Nearly all foods have a three-dimensional aspect to their color and the decision to reduce the data to two, or one, is obviously a value judgment.

Instrumentation is available to measure a single point on a reflection or transmission spectrum and one instrument gives a single-point reading in the red, yel-

low, blue, or green area. A single-point reading in the red region for a red beverage may well correlate with the overall color reading. Sometimes a ratio of two points is useful as, for example, the ratio 546/640 nm was used as the basis for redness in tomatoes for grading purposes. There are many examples of the use of ratios for color purposes in a manner similar to that for chemical analyses (7) (see also Chapter 19). The single-point "abridged" spectrophotometers have the advantages of simplicity and ruggedness but the readings cannot be transformed into fundamental units. The possible success of a single-point reading probably can be predicted from a reflection, or transmission, curve but each case should be judged on its own merits.

37.5 COLOR TOLERANCES

One of the most useful applications of color data is the setting of color tolerances for production control, stability, purchase specifications, etc. The color desired is located in color space and the allowable tolerances are plotted in one, two, or three dimensions in color space. Unfortunately it is not possible to specify a tolerance that is equally acceptable in all portions of color space because the eye is more sensitive to some colors. In the green area (Fig. 37-14), the ellipses are larger than the blue or red areas, indicating a greater sensitivity. An ellipse is preferred because it represents colors equidistant visually in all directions around a central point. Color tolerances may be plotted in three dimen-
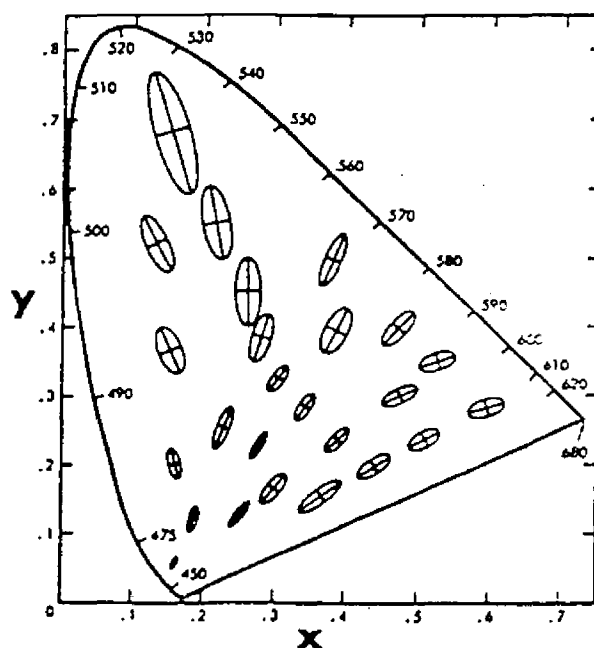
37-14
figure

Ellipses in color space representing the sensitivity of the human eye to various colors. The larger the ellipse, the more sensitive is the eye.
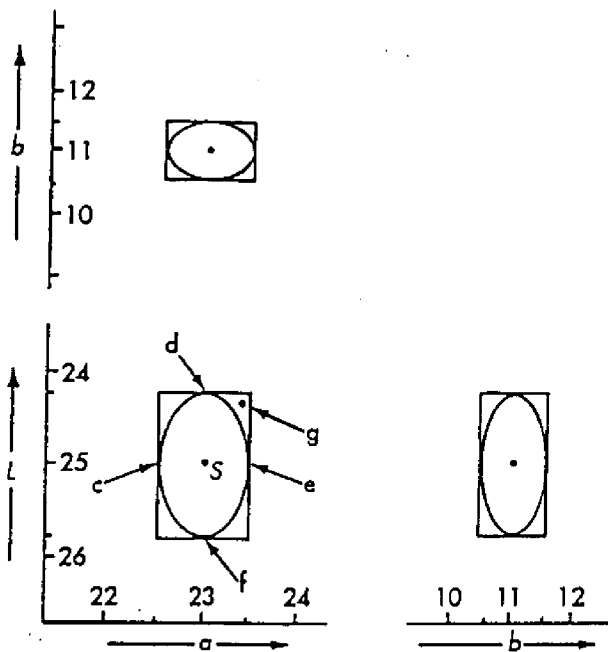
37-15 figure Examples of color tolerances plotted on a L a b diagram.

37-16 figure Diagram illustrating the visual impact of hue and chroma.

sions as shown in Fig. 37-15. An ellipse is preferred to a rectangle for color tolerances as shown in Fig. 37-15 where, if the central point is the desired color, the points c, d, e, and f would be within the tolerance if an ellipse was used. The point g would be within the tolerance using a rectangle and outside the tolerance if an ellipse was used.

The L* C* H* system was developed as a more convenient way to handle tolerances. It is a polar system with L* the same as in the L*a*b* system. The C* value represents the vector distance from the center of the color space to the measured color and is a measure of chroma (Fig. 37-16). The H* value is a measure of hue and is calculated in the same manner as θ. The L* C* H* system led to the development of the CMC color tolerancing system (16), which mathematically defines an ellipsoid around a standard color with the semiaxes corresponding to hue, chroma, and lightness. The ellipsoid, representing the volume of acceptance, automatically varies in size and shape depending on its position in color space. It also allows one to compensate for changes in sensitivity to lightness. The eye generally has greater acceptance for shifts in the lightness dimension than in hue or chroma. The tolerance ratio for lightness to hue or chroma is generally about 2:1. Figure 37-17 shows a CMC ellipsoid with a lightness:hue/chroma (l:h/c) ratio weighted at 2:1 and 1.4:1. Usually the amount of color difference that is considered acceptable is defined as a single commercial factor (cf). The cf can be varied to make the ellipsoid as large or as small as necessary.

37-17 figure Ellipsoid in the CMC color tolerancing system with a lightness:hue/chroma ratio weighted at 2:1 and 1.4:1.

The above paragraphs illustrate fairly sophisticated methods of specifying color tolerances. Whether this degree of accuracy is warranted in a given situation will differ with each application. Obviously, there is a tradeoff between the degree of accuracy, the cost of obtaining it, and the benefits to be derived from increased accuracy.

37.6 COLOR MEASUREMENT AS AN ANALYTICAL TOOL

A color measurement on the cut surface of, for example, a sweet potato can be done in a matter of seconds; thus it is a very attractive screening tool for plant breeders attempting to develop cultivars with high pigment content. Much research attention has been directed toward this aim and it does work with sweet potatoes (7). The Hunter a value correlates highly with the carotenoid content and actually shows more precision than a chemical analysis. The concept works because β-carotene comprises 80–90% of the total carotenoids. The procedure does not work with squash because the carotenoid content is distributed over six or more carotenoids. To handle colored products containing a series of pigments, it would be necessary to set up a regression equation with a factor for each important component. The complexities of this approach would suggest that it could be better handled by chemical analysis (see Chapter 19).

Color measuring instruments have potential as broad band filter photometers. Analytical instruments today that depend on absorptivity (See Chapter 26) usually employ a diffraction grating to obtain a narrow band of monochromatic light. However, some instruments use a glass filter that produces a narrow, medium, or broad transmission band, depending on the application. When the beam profile of the filter closely matches the profile of the chemical, the filter photometers can be very effective. Figure 37-18 shows the sensitivity of the eye to white light. Note the similarity of the curve representing the cones to the ȳ function in Fig. 37-6. This is not a coincidence since the Y coordinate was chosen such that all the luminosity would be in the Y parameter. This means that an instrument that can generate a Y or L reading can function as a broad band filter photometer. Actually it includes the
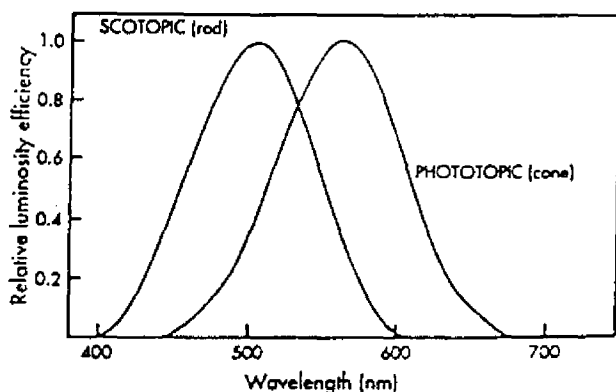
whole visible spectrum with a peak in the area of greatest sensitivity of the human eye. With samples that are primarily light or dark, the Y or L reading should function better than an absorptimeter based on a single wavelength. But there also may be a desire to use the a and b scales as indications of pigment content (17). Figure 37-19 shows a plot of L, a and b against concentration of cyanidin-3-glucoside (Cn-3-G), a common red pigment. The plots of both a and b show maxima between 0.2 and 5 mg of pigment. In these areas of confusion (17), it is difficult to tell where one is on the pigment scale. Eagerman et al. (18, 19) listed 11 color scales and they all showed areas of confusion. The change in direction is sometimes confusing to newcomers and it is because the calculations for a and b are tied into the L value. These areas of confusion can be removed by simply expanding the L scale, and a number of scale expansions have been suggested primarily for use with dark beverages. It is possible also to improve the prediction of pigment content from colorimetric data by linearizing the a or b scale. Figure 37-20 shows the a scale linearized for Cn-3-G. The equation needed to calculate the a value for Cn-3-G is:

$$\frac{100X}{Y^{1.60} - 0.5} \qquad [10]$$

Instrument responses for three other colorants, FD&C Red No. 1, FD&C Red No. 2, and FD&C Yellow No. 6, also are plotted using the same scale modication as for Cn-3-G. Note that there are no areas of confusion, but a scale linearized for Cn-3-G is not quite linear for the others. Similar scale calculations for a and b can be generated for any simple colorant or mixtures of colorants. In view of the complexities with a and b, a reader might be convinced that it would be simpler to use a simple absorptimeter.

37-18 figure The scotopic (rods) and photopic (cones) luminosity functions showing the sensitivity of the human eye to the spectral colors.
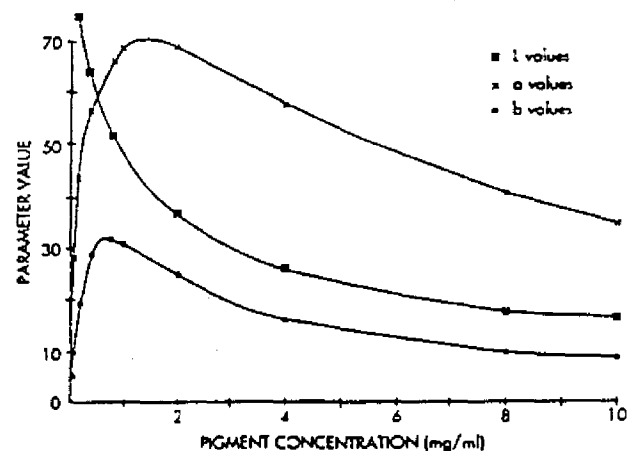
37-19 figure L a b values plotted against pigment concentration of a red pigment (Cn-3-G).
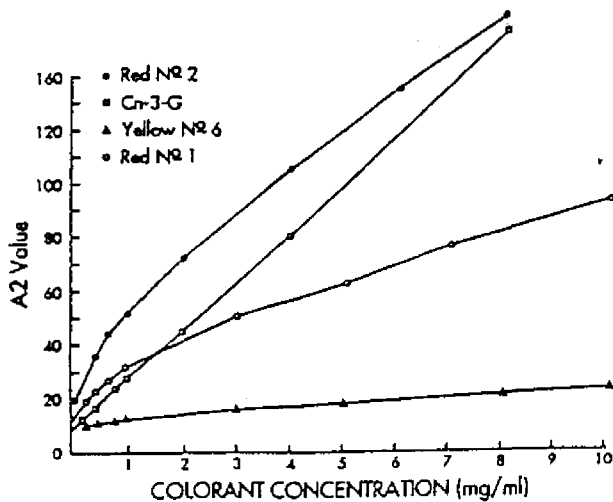
Plot of instrument response linearized for Cn-3-G against pigment content. Responses of three other colorants, using the same scale, also are plotted.

37.7 SUMMARY

Color can be defined as the interpretation by the brain of a light signal coming from a sample. The light signal enters the eye and impinges on the retina which has two types of light-sensitive cells. The rods are sensitive to lightness or darkness and the cones are sensitive to color. There are three types of cones: one sensitive to red light, another to blue, and the third to green. The responses of the three types of cones, for an average observer, to the spectral colors has been defined as the standard observer curves. The measurement of color depends on integrating the area under a reflection curve, or a transmission curve, in terms of the standard observer curves and the spectrum of the illuminant light. The data can be expressed in terms of the coordinates of a color solid. Color representation is a three-dimensional concept and a number of color systems have been suggested. Probably the best known in the paint, plastic, and textile industries is the CIE XYZ system, but the Lab, CIELAB, and CIELCH systems are receiving increased attention in these industries. With instrumental color measurement of foods, the Lab and the CIELAB systems have received the most acceptance. There also are a number of three-dimensional visual color atlases, of which the best known in the food area in the United States is the Munsell system.

When one is asked to "measure the color," what is really meant is to locate the coordinates of the color in three-dimensional space in a color solid. This can be done in three ways: visually by comparison with the chips in a color atlas, instrumentally by integrating the area under a curve, or by tristimulus colorimetry. A tristimulus colorimeter employs three glass filters

whose transmission curves duplicate the standard observer curves. A reading from each filter serves to locate a point in color space. Coordinates in any of the three types of approaches serve to identify unambiguously the color of a sample provided that the sample shows complete reflectance or complete transmittance. But usually food samples both transmit and absorb light energy. This may introduce some empiricism but it can be minimized by careful attention to sample presention. A number of instruments and mathematical equations have been developed to reduce the three parameters of color to two or one. There is some loss in accuracy with reduction in data but the tradeoff in terms of benefit and cost of data manipulation is a value judgment.

Color data in the food industry has been used in a variety of ways such as color tolerance specifications for purchase of ingredients and packages, process control, product stability, and overall consumer acceptability.

37.8 STUDY QUESTIONS

1. Describe the Munsell color system.
2. Define the "Standard observer" curves. How were they developed?
3. Describe how the CIE XYZ system was developed.
4. What do you understand by the term CIELAB?
5. What do you understand by the term "tristimulus colorimetry?"
6. Describe how a color tolerance can be set up in the Lab system.
7. Give an example of "reduction of data." Why is it sometimes desirable?
8. Can a tristimulus instrument for measuring color be used as a chemical absorptimeter? If so, how?

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